Amphiphilic poly-N-vinylpyrrolidone nanoparticles as carriers for non-steroidal, anti-inflammatory drugs: *In vitro* cytotoxicity and *in vivo* acute toxicity study

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**Abstract**

Polymeric nanoparticles were prepared from self-assembled amphiphilic N-vinylpyrrolidone polymers in aqueous media and evaluated as novel carriers of indomethacin, a non-steroidal, anti-inflammatory drug. It was determined that these nanoparticles could be created in spherical morphologies with sizes less than 100 nm, narrow size distributions and high indomethacin contents (up to 35\%) combined with high drug loading efficiencies (up to 95\%). In cytotoxicity tests using the human embryonic stem cell derived fibroblasts (EBF-H9) and hepatocellular carcinoma cells (HepG2), the indomethacin-loaded polymeric nanoparticles showed higher cell viability compared to that of free indomethacin at the same concentration. The median LD\(_{50}\) values, determined by the Litchfield–Wilcoxon method, were 55–70 mg/kg body weight depending on the polymer molecular design in both mice and rats. Based on the acquired results, these novel amphiphilic poly-N-vinylpyrrolidone nanoparticles can be considered as potential carriers for new, highly efficient, injectable drug delivery systems for hydrophobic drugs such as indomethacin.

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**Key words:** Poly-N-vinylpyrrolidone; Indomethacin; Amphiphilic; Nanoparticle; Toxicity; Drug delivery system

One of the crucial problems frequently encountered in pharmacology is the poor solubility of drugs in water and, as a consequence, their low bioavailability.\(^1\) To surpass this limitation, one of the most promising existing approaches is the inclusion of insoluble drugs into solubilizing nanoparticles. These nanoparticles should be colloidal, with sizes less than a micron, capable to release the drug in the body in a controlled manner or to carry the drug directly to its target. Controlled drug delivery offers better patient compliance by reducing the frequency of drug administration, reducing the side effects and leading to a better therapeutic control.\(^2\)–\(^7\)

The techniques that are currently used in order to obtain drug nanoparticles can be divided into two categories. The first is based on building up particles from dissolved drug molecules\(^8\)–\(^10\) whereas the second is based on breaking down of larger particles.\(^11\) e.g. media milling and high-pressure homogenization.\(^11\) Both synthetic polymers (polycrylates, polycaprolactones, polylactides and polylactidecopolymers with polyglycolides, etc.) and natural biopolymers (e.g. proteins, albumin, alginate, gelatin, chitosan) are used in the preparation of the pharmaceutical nanoparticles as potential formulations for site-specific drug delivery including drug targeting.\(^12\)–\(^15\)

In our previous study, self-assembled nanoparticles consisting of novel amphiphilic poly-N-vinylpyrrolidone derivatives possessing different molecular architectures were proposed as carriers for drug delivery.\(^16\) The direct control of nanoparticle structure, size and morphology combined with the high efficiency of hydrophobic drugs encapsulation and the simple, low-cost, scalable production technique makes them suitable for a variety of uses in biomedical and pharmaceutical formulations.\(^17\) For this reason, we prepared a series of nanoparticles and optimized them as drug delivery systems with high contents of an encapsulated model drug, such as indomethacin.\(^18\) Indomethacin, (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid), is a non-steroidal, anti-inflammatory drug with anti-pyretic and analgesic properties.\(^19\) The solubility and dissolution rate of indomethacin or other drugs with
poor solubility can be enhanced when combined with excipients with high solubility, for example solid dispersion.20 Our investigations showed that indomethacin can be effectively loaded and stabilized within the amphiphilic poly-N-vinylpyrrolidone nanoparticle hydrophobic core for extended periods of time.21 Despite of the interesting physicochemical characteristics of such nanoparticles, data on the in vivo and in vitro toxicity of polymeric carriers are rare. For this reason, in this study we focused on the investigation of the toxicity of the indomethacin-loaded amphiphilic poly-N-vinylpyrrolidone nanoparticles, as poly-N-vinylpyrrolidones are known to be well tolerated by organisms.22,23 In addition to the preparation of drug-loaded Amp-PVP nanoparticles and determination of their physicochemical characteristics, this work presents for the first time results of cytotoxicity tests using human embryonic stem cell derived fibroblasts (EBF-H9) cells and human liver hepatocellular carcinoma cells (HepG2). Moreover, acute in vivo toxicity studies have been carried out by determining the median lethal dose (LD50).

Methods

Materials

N-vinylpyrrolidone (VP), indomethacin (IMC), Dulbecco’s modified Eagle medium (DMEM), (3-(4,5-Dimethylthiazol-1-2-yl)-2,5-diphenyltetrazolium bromide(MTT), Trypan Blue solution, chloroform, dimethylsulfoxide (DMSO), fetal bovine serum (FBS), phosphate buffer saline (PBS), pyrene and all other chemicals used in this study were obtained from Sigma-Aldrich (USA) unless otherwise specified and used without further purification. The substrate for electron microscopy - 0.2% polyvinyl formal- was purchased from Merck (Germany). All solvents and components of buffer solutions were of analytical grade and used as received. A Milli-Q Plus System (Millipore, USA) was used for distilled–deionized water preparation.

Synthesis of Amph-PVPs

The amphiphilic N-vinylpyrrolidone polymers (Amph-PVP) were synthesized by using the originally developed two-step method described in our previous study.16 More specifically, Amph-PVP polymers differing in the molecular weight of the PVP hydrophilic fragment (4 and 8 kDa) and on the hydrophobic n-alkyl or di-n-alkyl fragment (samples PVP-OD4000, PVP-OD8000 and PVP-DD2800) were synthesized, characterized and used to prepare the IMC-loaded nanoparticles. The polymers, PVP-OD4000, PVP-OD8000 and PVP-DD28000, were synthesized in yields of 91%, 88% and 83% respectively. Their molecular weight (MW) was determined by steam osmometry using a Knauer osmometer (Germany) or alternatively by titration. The polydispersity of the prepared polymers was studied with high-performance liquid chromatography using a TSK Gel G4000PwxL column (Toso Co., Ltd., Japan). The Polydispersity index (PDI) values obtained were 1.19, 1.22 and 1.25 for PVP-OD4000, PVP-OD8000 and PVP-DD28000 respectively. The chemical structures of the polymers used in this study are presented in Figure 1. The critical aggregation concentration (CAC) of these PVP amphiphilic polymers was determined by steam osmometry using a Knauer osmometer (Germany) or alternatively by titration. The polydispersity of the prepared polymers was studied with high-performance liquid chromatography using a TSK Gel G4000PwxL column (Toso Co., Ltd., Japan). The Polydispersity index (PDI) values obtained were 1.19, 1.22 and 1.25 for PVP-OD4000, PVP-OD8000 and PVP-DD28000 respectively. The chemical structures of the polymers used in this study are presented in Figure 1. The critical aggregation concentration (CAC) of these PVP amphiphilic polymers was determined by pyrene fluorescence probe spectrometry.16

Preparation and characterization of IMC-loaded Amp-PVP nanoparticles

The amphiphilic N-vinylpyrrolidone polymeric nanoparticles loaded with indomethacin were prepared by the emulsification method with solvent evaporation. More specifically, different weight ratios of amphiphilic PVP and IMC were dissolved in a small volume of chloroform. Ultrasonic dispergation (Sonoplus HD 2070, Bandelin, Germany) was used to emulsify the mixtures in the aqueous phase. Subsequently, the organic solvent was distilled and the suspensions were concentrated by evaporation under reduced pressure (rotary evaporator Laborota 4010, Heidolph, Germany).

The IMC-loaded nanoparticle solutions were finally frozen and lyophilized using an Alpha 1-4LD freeze dryer system (Martin Christ GmbH, Germany). Thermo-gravimetric analysis of the freeze-dried nanoparticle powders confirmed the absence of organic solvent residues in the drug-loaded preparations.

Dynamic light scattering (Malvern Zetasizer Nano-ZS, Malvern, UK), with PBS (pH 7.4) as dispersion medium was used for the measurement of the particle size and zeta potential.21

The scattering intensity was used to calculate the size of the nano-scaled aggregates. The morphology of the Amph-PVP...
nanoparticles was determined by transmission electronic microscopy (TEM), using a JEM-2100 microscope (JEOL, USA).

The amount of IMC loaded in the nanoparticles was determined by measuring the UV absorbance at 318 nm using a Unico 2802 spectrophotometer (Uniko, USA). The IMC content introduced into the hydrophobic core of the nanoparticles was evaluated from the amount of drug incorporated in nanoparticles and the total weight of drug-loaded nanoparticle form using the following equation:

\[
\text{IMC content} (\%) = \frac{\text{weight of IMC in nanoparticles}}{\text{total weight of IMC loaded nanoparticles}} \times 100
\]

The Drug Loading Efficiency (DLE) was evaluated using the following equation:

\[
\text{DLE} (\%) = \frac{\text{weight of entrapped IMC in nanoparticles}}{\text{initial weight of IMC used}} \times 100
\]

Cells

Human embryonic stem cell derived fibroblasts (EBF-H9) cells and human liver hepatocellular carcinoma cell line (HepG2) from the D.I. Ivanovsky Institute of Virology of the Russian Academy of Science, Moscow, Russia were used for the cytotoxicity experiments. Fibroblasts mainly involved in the maintenance of the structural integrity of the connective tissue and production of the extracellular matrix proteins. Thus, they are widely distributed in the organism with unique functions. The selection of the EBF-H9 cells culture was based on previous studies demonstrating that human embryonic stem cell-derived fibroblasts could be used as a promising cell model for nanomaterials cytotoxicity screening. IMC is metabolized in the liver, therefore the hepatocellular toxicity of the polymer has been investigated. EBF-H9 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum, 1% penicillin-streptomycin solution at 37 °C under an atmosphere of 95% air and 5% CO₂ prior to experimentation. HepG2 cells were also grown in DMEM medium with addition of 10% FBS and 1% antibiotics at 37 °C in atmosphere of air containing 5% CO₂ in a humidified CO₂ incubator.

Animals

BALB/C mice (males and females, age: 6 to 7 weeks; body weight: 20–22 g) and Wistar rats (males and females, age: 7 to 8 weeks; body weight: 150–180 g) were obtained from Branch of the M.M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences in Pushchino (Moscow Region, Russia). Animals were acclimatized for 2 weeks before the experiments. They were housed in plastic cages (4 animals/cage for rats and 5 animals/cage for mice) under conditions of controlled temperature maintained at 20–23 °C, humidity of 60–70%, ventilation (10–12 times/h) and were kept on a 12 h light/dark cycle. Free access to commercial food and water was allowed. All animal experiments were performed in compliance with the local ethics committee regulations and guidelines on animal welfare and in compliance with EU Directive 2010/63/EU for animal experiments. All animals were humanely treated during the experiment.

In vitro cytotoxicity test

Cell viability was chosen as cytotoxicity parameter and determined using the MTT as well as the trypan blue exclusion assay.

For the MTT tests both cells types (10⁴ cells/well) were cultured in a 96-well plate in 5% CO₂ atmosphere at 37 °C and exposed to varying concentrations of IMC-free or IMC-loaded Amph-PVP nanoparticle solutions for 24 and 48 h. Cells treated with free indomethacin in the absence of polymer nanoparticles were also studied for comparison, while cells treated only with medium were used as the control group. After removing the supernatant of each well and washing twice with PBS, 20 μl of an MTT solution (5 mg/ml in PBS) and 100 μl of medium were introduced to the wells. Upon incubation for a period of 4 h, the resultant formazan crystals were dissolved in DMSO (100 μl) and the absorbance intensity was measured on a microplate reader (Immunochrome 2100, USA) at a wavelength of 570 nm. All experiments were performed in triplicate and the relative cell viability (%) was expressed as a percentage relative to the control cells.

For the trypan blue exclusion tests both type of cells were seeded into 96-well plates (10⁴ cells per well) in 5% CO₂ air atmosphere at 37 °C and were then treated with various concentrations of IMC-free or IMC-loaded Amph-PVP nanoparticles, or with free indomethacin for 24 and 48 h.

The number of viable cells was counted on a hemacytometer with the help of the trypan blue dye (0.4% solution) after cells trypsinization. The viability of the control (untreated cells) was regarded as 100%. Three repeats of counting were performed for each well and each experiment was repeated three times.

In vivo acute toxicity study

In order to investigate the acute toxicity of the IMC-loaded Amph-PVP nanoparticles, the median lethal dose (LD₅₀) was determined using male and female BALB/C mice and Wistar rats. The animals were divided into groups consisting of 10 mice (5 females and 5 males) or 8 rats (4 females and 4 males) each. Experimental animals were intraperitoneally injected with fixed doses of IMC-loaded Amph-PVP nanoparticles containing 1, 10, 30, 50, 70, 90, 100 mg/kg body weight of indomethacin or with the same dose of free IMC and lethal response was observed for 14 days after administration. Doses were chosen to provide the highest toxicity dose approaching that of the reported LD₅₀ for IMC which is about 13 mg/kg body weight. A two-step approach was used to estimate the LD₅₀. The approximate LD₅₀ values were initially obtained by the Deichmann and LeBlanc method, and were then refined using the Litchfield and Wilcoxon method.

Statistical analysis

The results were expressed as median or mean ± standard deviation (SD). The statistically significant differences between
several parallel experiments or between experimental groups were analyzed by the independent Student’s t test for paired samples. A level of confidence of \( p < 0.05 \) was applied for the statistical significant.

**Results**

Several amphiphilic derivatives of poly-N-vinylpyrrolidone-varying on both the PVP hydrophilic moiety and the terminal n-alkyl or di-n-alkyl moiety were synthesized and thoroughly investigated in our previous works.\(^{16,17}\) The influence of their structure on the self-assembly in aqueous media as well as on the physicochemical properties of the prepared polymeric nanoparticles were revealed. In addition, the efficiency of indomethacin encapsulation was found to depend on both the polymer structure and the encapsulation conditions while, the \textit{in vitro} controlled release of indomethacin was also comprehensively investigated.\(^{18,21}\) Herein, we present for the first time a comparative study of the \textit{in vitro} cytotoxicity and \textit{in vivo} acute toxicity of a series of indomethacin loaded Amph-PVP polymeric nanocarriers with the aim to fully investigate their intrinsic characteristics as drug delivery systems in order to exploit them to their full potential.

**Synthesis of Amph-PVPs**

The structures and main characteristics of the Amph-PVP polymers (PVP-OD4000, PVP-OD8000 and PVP-DD\(_2\)8000) synthesized and utilized in the current study are presented in Figure 1 and Table 1. All polymers were synthesized in high yields and good polydispersity. More importantly, all polymers possessed rather large hydrophilic and hydrophobic blocks and could self-assemble in aqueous media at concentrations higher than their critical aggregation concentration (CAC) to form nano-scaled spherical particles with a hydrophobic inner core and a hydrophilic outer shell. The CACs of all three synthesized polymers were determined by pyrene fluorescence probe spectrometry and were all found in the micromolar range, \textit{i.e.} sufficiently low for the nanoparticles to maintain their self-assembled state during investigations (Table 1).

**IMC-loaded Amph-PVP nanoparticles**

In previous studies we demonstrated that hydrophobic drugs, such as indomethacin (Figure 1) can easily be incorporated into the hydrophobic core during the self-assembly process of the polymeric nanoparticles.\(^{18,21}\) In this study, IMC-loaded nanoparticles were prepared using the amphiphilic polymers PVP-OD4000, PVP-OD8000 and PVP-DD\(_2\)8000 via the solvent evaporation technique to study the influence of polymer structure on nanoparticle properties and biocompatibility. Hollow nanoparticles were also prepared in the absence of IMC for comparison. The freeze-dried IMC-loaded or hollow nanoparticles were resuspended in PBS for further characterization. The composition and properties of prepared nanoparticles are presented in Table 2.

Dynamic light scattering studies were performed in order to investigate the average hydrodynamic size and surface charge of the nanoparticles. The average size of the hollow Amph-PVP nanoparticles was found to be smaller than 150 nm and the size distribution was narrow and mono-dispersed (Figure 2, A). Particle size increased with the increase of the molecular weight of the PVP fragment and of the amphiphilic polymer concentration in solution as can be seen in Table 3. Transmission electron microscopy confirmed that all prepared nanoparticles have spherical morphology (Figure 3).

As also observed in our previous studies, loading IMC on the Amph-PVP nanoparticles at polymer concentrations near CAC, was found to introduce a slight increase of their size.\(^{18}\) \textit{Vice versa}, at high polymer concentrations, introduction of the hydrophobic drug in the system was found to cause its compacting and decrease of IMC-loaded particles size to less than 100 nm. However, the size distribution of nano-aggregates in both cases was almost identical with the narrow distribution observed before IMC loading (Figure 2).

The amount of indomethacin introduced in the Amph-PVP nanoparticles and the drug loading efficiency (DLE) was effectively controlled by the structure of the amphiphilic polymer (\textit{i.e.} the relative size of the hydrophobic and hydrophilic blocks) and by the initial weight ratio between the amphiphilic polymer and IMC (Table 4). The content of indomethacin in the Amph-PVP nanoparticles and DLE was found to increase with the increase of the hydrophobic fragment moiety in the amphiphilic macromolecule. Furthermore, the efficiency of IMC entrapment effectively decreased when the initial feed drug/polymer ratio exceeded the 0.5/1.0 ratio. This ratio can therefore be considered as optimal for maximum IMC content in the Amph-PVP nanoparticles and maximum DLE (up to 90–95%, Table 4).

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**Table 1** Characteristics of the Amph-PVP polymers.

<table>
<thead>
<tr>
<th>Amphiphilic polymer</th>
<th>Molecular weight, Da</th>
<th>Hydrophobic fragment</th>
<th>PDI*</th>
<th>CAC**, ( \mu M/l )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP-OD 4000</td>
<td>4000</td>
<td>n-octadecyl</td>
<td>1.186</td>
<td>6.2.</td>
</tr>
<tr>
<td>PVP-OD 8000</td>
<td>8000</td>
<td>n-octadecyl</td>
<td>1.218</td>
<td>9.3.</td>
</tr>
<tr>
<td>PVP-DD(_2) 8000</td>
<td>8000</td>
<td>di-n-dodecyl</td>
<td>1.252</td>
<td>15.4.</td>
</tr>
</tbody>
</table>

* PDI: Polydispersity index.
** CAC: Critical aggregation concentration.

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**Table 2** Characteristics of IMC-loaded Amph-PVP polymeric nanoparticles (mean ± SD, \( n = 3 \)).

<table>
<thead>
<tr>
<th>Amphiphilic polymer</th>
<th>IMC/polymer weight ratio</th>
<th>Particle size, nm</th>
<th>Zeta potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP-OD 4000</td>
<td>0.00: 1.00</td>
<td>118.4 ± 5.2</td>
<td>−1.32 ± 0.54</td>
</tr>
<tr>
<td>PVP-OD 4000</td>
<td>0.25: 1.00</td>
<td>78.1 ± 2.7</td>
<td>+1.29 ± 0.23</td>
</tr>
<tr>
<td>PVP-OD 8000</td>
<td>0.00: 1.00</td>
<td>134.7 ± 4.8</td>
<td>−2.76 ± 0.63</td>
</tr>
<tr>
<td>PVP-OD 8000</td>
<td>0.25: 1.00</td>
<td>92.1 ± 3.4</td>
<td>+0.75 ± 0.36</td>
</tr>
<tr>
<td>PVP-DD(_2) 8000</td>
<td>0.00: 1.00</td>
<td>152.2 ± 4.4</td>
<td>−4.18 ± 0.38</td>
</tr>
<tr>
<td>PVP-DD(_2) 8000</td>
<td>0.25: 1.00</td>
<td>98.4 ± 5.4</td>
<td>−1.62 ± 0.45</td>
</tr>
</tbody>
</table>

Polymer concentration 20 \( \mu M/l \).
In vitro cytotoxicity of IMC-loaded Amph-PVP nanoparticles

Within the framework of the in vitro cytotoxicity experiments, cell viability in the presence of different IMC-loaded Amph-PVP nanoparticles preparations was compared to that of free, non-encapsulated IMC and the control after 24 and 48 h of incubation in the culture of EBF-H9 cells and HepG2. Both cell types were tested with equal concentrations of Amph-PVP nanoparticles, which were either loaded with IMC or hollow. The cytotoxicity of free indomethacin was also studied for comparison.

The percentage of viable cells in the suspensions was determined by both the MTT and trypan blue exclusion assays. Figures 4-9 demonstrate EBF-H9 and HepG2 cell viability in presence of IMC-loaded PVP-OD4000, PVP-OD8000 and PVP-DD28000 nanoparticles, the corresponding hollow nanoparticles created in the absence of IMC, and free indomethacin. Control was untreated cells (without any treatment) and their viability was counted as 100% for each experiment and other results were counted relatively to these 100%.

The percentage of viable cells in the suspensions was determined by both the MTT and trypan blue exclusion assays.

The unloaded hollow polymer nanoparticles did not reveal any significant cytotoxicity for either cell type or assay used during this investigation. These findings demonstrated the good biocompatibility of the polymer carriers themselves that resulted in cell viability of over 90% at the highest polymer concentrations. For all IMC-loaded Amph-PVP nanoparticles, the cytotoxicity evaluations by the MTT and the trypan blue exclusion assays using both EBF-H9 and HepG2 cells showed similar effects (Figures 4-9).

In vivo acute toxicity of IMC-loaded Amph-PVP nanoparticles

The values obtained for lethal doses are presented in Table 5. The median lethal doses (LD_{50}) of IMC-loaded Amph-PVP nanoparticles as determined by the Litchfield–Wilcoxon method ranged between 55 to 70 mg/kg body weight, that is several times higher than the evaluated LD_{50} for free IMC (15 mg/kg bw). When comparing the acquired LD_{50} values between different sets of experiments (Table 5) no difference in acute toxicity was observed for male and female animals (p > 0.05). Moreover, no statistically significant difference was observed (p > 0.05) in acute toxicity of IMC-loaded Amph-PVP nanoparticle preparations between rats and mice. In accordance to the obtained LD_{50} values, all tested polymeric nanoparticle IMC preparations can be referred to moderately toxic substances, class 3, according to the Hodge and Sterner scale.

![Figure 2. Size distribution of PVP-OD8000 (A) and IMC-loaded PVP-OD8000 (B) polymeric nanoparticles. PVP-OD8000 concentration 20 μM/l, IMC content 25 w/w %.

Table 3

Average diameter and polydispersity index (PDI) of the amphiphilic PVP nanoparticles depending on polymer structure and concentration.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>C_{p} ~ CAC*</th>
<th>C_{p} = 1.0 mg/ml</th>
<th>C_{p} = 5.0 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D***, nm</td>
<td>PDI***</td>
<td>D, nm</td>
</tr>
<tr>
<td>PVP-OD 4000</td>
<td>43 ± 4.1</td>
<td>0.181 ± 0.021</td>
<td>154 ± 6.9</td>
</tr>
<tr>
<td>PVP-OD 8000</td>
<td>62 ± 4.8</td>
<td>0.208 ± 0.040</td>
<td>172 ± 10.1</td>
</tr>
<tr>
<td>PVP-DD28000</td>
<td>86 ± 7.2</td>
<td>0.244 ± 0.034</td>
<td>218 ± 8.6</td>
</tr>
</tbody>
</table>

* C_{p} – polymer concentration, CAC – critical aggregation concentration.
** Nanoparticles diameter (mean ± SD).
*** Nanoparticles polydispersity index (mean ± SD).
Polymeric nanoparticles consisting of amphiphilic poly-N-vinylpyrrolidones were found in previous studies to be promising carriers for creating highly-effective IMC delivery systems. However, so far, very few studies concerning the biocompatibility and toxicity of such nanoparticles have been reported. For this purpose, IMC-loaded Amph-PVP nanoparticles were prepared through self-assembly of the amphiphilic macromolecules in aqueous media with simultaneous entrapment of IMC in the nanoparticle hydrophobic inner core.

The results of this investigation confirm that the prepared IMC-loaded Amph-PVP nanoparticles have spherical morphology, sizes lower than 100 nm and monomodal narrow size distribution. The size of the Amph-PVP nanoparticles was found to increase with the molecular weight of the hydrophilic PVP fragment of the amphiphilic macromolecules and decrease when larger terminal hydrophobic fragments were introduced in the polymer structure. The sizes of the IMC-loaded polymer nanoparticles were also found to be influenced by the amount of drug utilized to prepare the nanoparticles. Such results can be attributed to the differences in the hydrophilic/hydrophobic balance within the structures of the different amphiphilic polymers used in this study, resulting in altering of the hydrophobic interaction forces between the macromolecules and IMC.

Optimal IMC entrapment was reached as the initial feed ratio of IMC to polymer was increased to 0.5/1.0 during the preparation of the polymeric nanoparticles, as would be expected considering the rather strong hydrophobic nature of the drug. For example, IMC content of about 32% was obtained for IMC-loaded Amph-PVP nanoparticles when the feed weight ratio of IMC to polymer was 0.5/1. In conclusion, the main factor influencing IMC encapsulation within the Amph-PVP nanoparticles is the hydrophobic interaction between IMC molecules and the polymer nanoparticle hydrophobic core. Consequently, most of the drug IMC is expected to be entrapped within the nanoparticle core and this process to be more efficient for polymers with larger hydrophobic moieties. These data fully agree with previous experiments on the in vitro release behavior of similar IMC-loaded Amph-PVP nanoparticles, when some initial burst effects were detected at high feed ratio of IMC to polymer and were attributed to residual amounts of IMC linked with the polymer nanoparticle hydrophilic outer shell while, no burst effects were observed at lower content of IMC when all drug was entrapped within the nanoparticle hydrophobic core, providing a slow and steady IMC release into the medium.21

From the results of the in vitro cytotoxicity trypan blue exclusion and MTT test on two different cell types (EBF-H9 and HepG2), two main factors influencing cell viability were
Firstly, the direct interaction between IMC molecules and cells were found to be significantly reduced when IMC was predominantly loaded in the hydrophobic inner core of the polymeric nanoparticles. On the other hand, the outer shell of the nanoparticles consisting of hydrophilic PVP chains was also found to reduce the interaction both between nanoparticles themselves and between nanoparticles and cells by forming something like a "stealth" layer. Additionally, cell viability could also be maintained due to the controlled and prolonged IMC release profile from the drug-loaded Amph-PVP nanoparticles, which results in decreasing the amount of IMC interacting with cells in a predetermined period of time.

The most commonly accepted release model assumes that after injection into the blood stream, nonionic, intact polymeric self-assembled nanoparticles carry the drug molecules until being internalized via endocytosis after nonspecific association with the cell membrane and intracellularly release the drug. Nevertheless, this mechanism has not been fully experimentally confirmed up to date and the pathway for cellular internalization of polymeric micelle-type nanoparticles and entrapped hydrophobic drug molecules remains unclear. In previous investigations, we demonstrated that Amph-PVP polymers could be easily incorporated into lipid bilayers by their hydrophobic fragment, forming an outer shell of hydrophilic PVP chains which resulted in the effective modification of liposome membranes by increasing their stability. Taking into account the dynamic instability of polymeric self-assembled nanoparticles we can consider a similar possible mechanism for the release of hydrophobic IMC molecules from the Amph-PVP nanoparticle core to cell lipid bilayers. After interaction of the Amph-PVP nanoparticles with the cell membrane, IMC molecules loaded within the hydrophobic core can be effectively transferred to the cell membrane, from which they can then be diffused or endocytosed to the intracellular targets, including specific structures. The cellular uptake of IMC may be faster than that of Amph-PVP due to the strong hydrophobic nature of the drug and the amphiphilic nature of the polymer, so two separate cell entry pathways for IMC and the amphiphilic polymer have to
be assumed. It is also possible that the Amph-PVP polymers will not enter the cell but rather return back to the blood stream or that IMC could be released due to the interaction with membrane lipids and enter the cells separately. As a result, Amph-PVP nanoparticles could act as transporters of the hydrophobic agents via membrane lipid interactions. This proposed mechanism remains to be confirmed and it is the main object of our further investigations. It is worth mentioning that results obtained for mono-methoxypoly(ethylene glycol)-block-poly(D,L-lactic acid) micelles are in accordance with such a mechanism of polymer nanoparticle interaction with cell membranes.

The results of the in vitro cytotoxicity test using the MTT and the trypan blue exclusion assay with both EBF-H9 and HepG2 cells, confirmed that, in contrast to free indomethacin, IMC-loaded Amph-PVP nanoparticles did not induce any noticeable cytotoxicity against normal human fibroblast cells, which can be considered as evidence for the increased IMC biocompatibility when hidden in the core of polymer nanoparticles.

Indomethacin is a widely used, non-steroidal, anti-inflammatory drug with antipyretic and analgesic properties commonly used for the treatment of patients with rheumatoid arthritis. The therapeutic effective dose of IMC is about 25–75 mg at once in humans and the known intraperitoneal LD50 of IMC in rats is about 13 mg/kg body weight, which is in full accordance with the free IMC LD50 values obtained in the current study. The LD50 of IMC-loaded Amph-PVP nanoparticles determined by Litchfield–Wilcoxon method were in the range of 55 to 70 mg/kg body weight in BALB/C mice and Wistar rats, with no significant difference between sex and species sensitivity observed in the tested preparations. Based on our results, we can conclude that the IMC-loaded Amph-PVP nanoparticle preparations with IMC content of more than 30% represent an efficient injectable drug carrier for IMC. Further experiments considering the use of other routes of exposure such as intravenous injection or oral administration are in progress in order to fully support the moderate toxicity of indomethacin-loaded Amph-PVP nanoparticles.
From the results obtained within this study, we propose that nanoparticles consisting of N-vinylpyrrolidone amphiphilic polymers can be considered to be novel, efficient and biocompatible nano-scaled carriers for potential injectable drug delivery systems aimed for the controlled release of active substances. In the case of anti-inflammatory drugs, this targeted delivery could lead to decreased doses of drug, better efficacy and reduced side effects at the gastric, hepatic and cardiovascular level.

Figure 8. Cytotoxicity of unloaded Amph-PVP nanoparticles during incubation with EBF- H9 cells for 48 h based on the MMT test. Cell survival values are expressed as the percentage of control values. Values are mean ± SE of three independent experiments. *P < 0.05 in comparison to the control.

Figure 9. Cytotoxicity of unloaded Amph-PVP nanoparticles during incubation with HepG2 cells for 48 h based on the trypan blue test. Cell survival values are expressed as the percentage of control values. Values are mean ± SE of three independent experiments. *P < 0.05 in comparison to the control.

Table 5
IMC LD₅₀ toxicity index in IMC-loaded Amph-PVP nanoparticle preparations for single intraperitoneal administration in BALB/C mice and Wistar rats.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Sex*</th>
<th>IMC content, %</th>
<th>Median LD₅₀ toxicity index** in mice, mg/kg bw IMC</th>
<th>Median LD₅₀ toxicity index** in rats, mg/kg bw IMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free IMC</td>
<td>Male</td>
<td>100.00</td>
<td>13 ± 3</td>
<td>15 ± 3</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>100.00</td>
<td>14 ± 2</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>PVP-OD 4000</td>
<td>Male</td>
<td>32.40</td>
<td>64 ± 3</td>
<td>66 ± 5</td>
</tr>
<tr>
<td>PVP-OD 8000</td>
<td>Male</td>
<td>32.96</td>
<td>69 ± 4</td>
<td>71 ± 4</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>32.96</td>
<td>67 ± 6</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>PVP-DD2 8000</td>
<td>Male</td>
<td>31.44</td>
<td>56 ± 4</td>
<td>55 ± 5</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>31.44</td>
<td>58 ± 5</td>
<td>59 ± 7</td>
</tr>
</tbody>
</table>

* No statistically significant species- and sex-differences in toxicity observed (p > 0.05).
** Evaluated against pure IMC content in nanoparticles.
References


