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# Nanohydrogel with *N,N′*-bis(acryloyl)cystine crosslinker for high drug loading

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ABSTRACT

Substantially improved hydrogel particles based on poly(*N*-isopropylacrylamide) (pNIPA) have been obtained. First, as a result of replacing commercially available *N,N′*-bis(acryloyl)cystamine (BAC), the crosslinker, with acryloyl derivative of cystine containing a carboxylic group (BISS), the hydrogel particles acquired improved stability *vs.* ionic strength and allowed further chemical modification of the chains, including the attachment of drug molecules. Next, a redox-initiated aqueous precipitation polymerization *via* the semi-batch method was used. This led to substantially increased BISS content and diminished size of the nanoparticles that made them suitable to an endocytic process. In addition, the obtained nanogels revealed high loading capacity of anticancer drug *vs.* dry gel (circa 16%) and they exhibited much better stability and enhanced drug release under the typical conditions existing in cancer cells. Size of obtained nanogels was investigated by dynamic light scattering (DLS). It appeared that nanoparticle size was in the range from ca. 40 to 200 nm. In 0.01 M solution of glutathione (GSH) the -S-S- bonds were reduced and the nanogel particles were degraded. This could be seen in obtained SEM and TEM micrographs. The cytotoxicity investigation against the HeLa cells showed that DOX loaded nanogels were more cytotoxic ($IC_{50}$ = 0.51 μM) than free DOX ($IC_{50}$ = 0.83 μM), while unloaded nanogels did not inhibit proliferation of the cells. It was also found that the nanogels loaded with DOX reached a high intracellular concentration in HeLa cells just after 2 h while free DOX needed 6 h for that.



## 1. Introduction

Nanogels are particles made of polymer chains appropriately linked and filled with a solution. They may be smaller than 100 nm. Compared to regularly sized hydrogels, nanogels can be injected and usually quickly respond to changes in environmental conditions. These features make them very interesting candidates for drug carriers. Potentially, a very useful property of nanogels is their sensitivity to changes in T and pH. Among thermoresponsive gels, the micro- and nanogels based on *N*-isopropylacrylamide (NIPA) are of increasing interest. These hydrogels undergo a drastic drop in volume when temperature exceeds 32 °C. pNIPA is effectively non cytotoxic in many medical applications (Malonne et al., 2005; Hathaway et al., 2017; Vihola et al., 2005). However, there is one disadvantage of using these gels; they cannot be degraded because the used crosslinkers are resistant to cleavage procedures. To eliminate this limitation the -S-S- group was used within the crosslinking molecules. The presence of -S-S- groups enables the split of the cross-linkers what leads to disintegration of the gel particles and to release of the absorbed molecules (Tian et al., 2016; Zhang et al., 2016a; Jin et al., 2015; Péreza et al., 2015). The split of -S-S- group can be achieved after treating with a reducing agent, *e.g.* glutathione (GSH), concentration of which is higher in intracellular environments compared to the extracellular one (Elzes et al., 2016; Zhang et al., 2016b; Li et al., 2016; Meng et al., 2009) and also is *ca.* 4-fold higher in most cancer cells than in the normal cells (Gamcsik et al., 2013). Generally, the environment in tumor tissues is stronger reducing, more hypoxic and more acidic compared with normal tissues. Some examples of microgels that exhibit the degradability were synthesized using *N,N′*-bis(acryoyl)cystamine (BAC) that is offered on the market (Tian et al., 2016; Zhang et al., 2016a; Jin et al., 2015; Péreza et al., 2015; Zhan et al., 2015). In those papers BAC was used as the crosslinker in nano- and microgels based on 2-(2-methoxyethoxy)ethyl methacrylate and oligo(ethylene glycol)

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methacrylate (Tian et al., 2016), carboxymethyl chitosan (Zhang et al., 2016a), methacrylic acid (Jin et al., 2015), a mixture of *N*-isopropylacrylamide, *N*-hydroxyethylacrylamide and tert-butyl 2-acrylamidoethyl carbamate (Péreza et al., 2015), and a mixture of *N*-isopropylacrylamide and acrylic acid (Zhan et al., 2015). Another limiting characteristics of pNIPA gels is that they are insensitive to pH changes and they are unstable under physiological conditions (moderate and high ionic strength).

Precipitation polymerization is usually employed in preparation of pNIPA-based gels of colloidal-size. Under such conditions, in the absence of surfactant, the size of pNIPAM microparticles is at the level of 1000 nm; this is a too big size to enable the possibility of using the microgel as a drug carrier. The size of particles has crucial impact on the endocytic process. The optimal diameter for the nanoparticle endocytic uptake into cells sits in the range of 25–100 nm (Zhang et al., 2009). When a surfactant, such as sodium dodecyl sulfate (SDS), is added to the reaction mixture the size of the synthesized particles is below 300 nm (Jones and Lyon, 2000).

A disadvantage of the precipitation polymerization is that this method utilizes relatively high temperatures (70 °C) to form a radical from a persulfate salt. The presence of the -S-S- group during micro- and nanogel synthesis complicates the process, since this bond may be cleaved and sulfur radicals may be formed. The presence of sulfur radicals may result in the creation of sulphide groups that are nonreducible and therefore make the polymeric net more resist to degradation (Senning, 1971). To lower synthesis temperature an accelerator of decomposition of the persulphate anion was used. A commonly used accelerator is *N,N,N′,N′*-tetramethylethylenediamine (TEMED). TEMED can generate more sulfate radicals than the thermal decomposition. The pNIPA synthesis done at slightly increased temperature and in the presence of TEMED and a persulphate salt led to the formation of microparticles without self-crosslinking (Hu et al., 2011; Chen and Sajjadi, 2014a,b).

By employing TEMED with a persulphate salt and lowering the synthesis temperature we eliminated the self-crosslinking and could introduce just one crosslinker. It was bisacryloyl derivative of cystine (*N,N′*-bisacryloylcystine, BISS). We aimed at obtaining nanogels of much better properties and usefulness compared to not fully satisfying microgels synthesized by us earlier (Mackiewicz et al., 2015). The gel microparticles obtained earlier were too big (400–900 nm) and efficiency of drug loading was rather low.

## 2. Experimental

### 2.1. Chemicals

*N*-isopropylacrylamide (NIPA, 97%), acryloyl chloride (96%), potassium persulfate (KPS, 99.99%) and reduced L-glutathione (GSH, 98%) were supplied by Aldrich. NaOH (99%), HCl (35–38%) and L-cystine (98,5%) were purchased from POCh.

NIPA was purified by recrystallization from (9:1) mixture of benzene and hexane. High purity water was used for preparation of all solutions. Water was purified with a Hydrolab/HLP purification system; the final conductivity of water was 0.056 $\mu S\,cm^{-1}$.

### 2.2. Synthesis of BISS

A method presented in patent (Shand et al., 2005) was used in the synthesis of the linker with disulphide bridges. Briefly, to a suspension of L-cystine (2.70 g, 11.2 mmol) in methanol (70 mL), sodium hydroxide (2 g, 50 mmol) was added and the mixture was clarified. Then 2.20 mL (27.2 mmol) of acryloyl chloride was added drop by drop at 0 °C, water bath was removed and the reaction mixture was agitated at RT for 4 h. The solid particles were filtered out using a celite pad. The obtained filtrate was added drop by drop to intensively agitated cold diethyl ether. A precipitate was formed. It consisted of disodium salt of *N,N′*-bisacryloylcystine. The obtained salt was filtered out, washed with diethyl ether and vacuum dried at 30–45 °C.

### 2.3. Nanogel synthesis

Nanogels were prepared by employing the precipitation polymerization (Galaev and Mattiasson, 2008) *via* the semi-batch method (Ramil et al., 2013). The reactor (a 250 mL three-necked flask equipped with a magnetic stirrer, reflux condenser, inlet and outlet of inert gas) was initially charged with 40 mL of an aqueous solution of KPS and BISS. Temperature was increased to 55 °C while the reaction mixture was purged with argon and stirred (1400 rpm). Next, the argon line was lifted to sit well above the solution surface, the agitation speed was reduced to 700 rpm. A solution of NIPA and 10-μL of TEMED in 10 mL of deionized water was prepared in another three-neck flask. Then it was added dropwise to 40 mL of KPS and BISS solution. Prior to addition, the NIPA and TEMED solution was heated up at 55 °C, gently stirred and deoxygenated with argon for 30 min. Finally the argon line was lifted to sit above the surface of the monomer solution and the mixing was turned off. The total concentration of BISS and NIPA in the reaction mixture was set at 100 mM. The BISS mole fraction equaled 5%. The total concentration of KPS was 5 mM. The rate of dosing NIPA monomer (with TEMED) was 0.1 mL $min^{-1}$. The process of monomer addition lasted 100 min. The solution of BISS and initiator was initially clear and transparent but became translucent to opaque when the polymerization advanced. The reaction was carried out under argon blanket and lasted 4 h. Fig. 1 presents a scheme of the synthesis and the structure of synthesized nanogel net.

To purify the nanogels they were placed in a Spectra/Por® dialysis bag of 10 kDa molecular weight cutoff. The dialysis was done with 5 L of water for 14 days at ambient temperature. Water was changed each day.

### 2.4. Instrumental

#### 2.4.1. Dynamic light scattering

A Malvern Zetasizer instrument (Nano ZS, UK) equipped with a 4 mW Helium-Neon laser with light wavelength of 632.8 nm was used to determine hydrodynamic diameter of the nanogel particles. The employed scattering angle was 173°. Before measurements the nanogel suspensions were filtered and kept at designated temperatures for 5 min. The Zetasizer instrument allowed also the determination of nanogel Zeta potential. These measurements were done with an U-folded capillary cell and two Au electrodes. Zeta-potential was in fact calculated from electrophoretic mobility. For this purpose the analyzer software used the equations of Henry and Smoluchowski.

#### 2.4.2. Scanning electron microscopy (SEM and TEM) and fluorescence microscopy

A Merlin (Zeiss) instrument working at 3 kV was used to take SEM micrographs. Before measurements nanogel samples were dried at 50 °C. Then they were coated with a 3-nm layer of vacuum deposited Au-Pd alloy. This was done with a Mini Sputter Coater (Polaron SC7620).

A Libra 1200 (Zeiss) instrument was used to take TEM micrograms. The samples were prepared by placing a drop of aqueous nanogel on a formvar-coated copper grid and allowing them to dry in air.

The fluorescence photos were taken at 200× magnification with an Olympus BX60 microscope with a WBV fluorescence unit, equipped with a DELTA Optical DLT-Cam PRO 5 MP camera.