Novel Functionalized Nanohydrogels, Synthesis and some Applications

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Abstract. A functionalized nanohydrogels have been synthesized by two step procedure. The first step implies an inverse microemulsion polymerization of p-nitro phenol acrylate (NPA) and N-isopropylacrylamide (NIPA) using Aerosol (AOT) as a surfactant and ethylene glycol dimethacrylate (EGDMA) as a crosslinking agent. The polymerization reaction was performed in presence of an oil-soluble salt to reduce the dimensions of the micellar diameter. The second step includes a chemical functionalization by nucleophilic substitution reaction over the carbonyl groups. The average particle diameter and the particle size distribution of the nanohydrogels were measured in acetone at 25°C by quasielastic light scattering (QLS) showing average diameter of 22 nm. The nanogels were characterized by FTIR-ATR, ¹H NMR, UV-vis spectroscopy and DSC.

1. Introduction
For the last decades the breakthrough occurred in synthesis and modification area of macromolecules has enabled to replace conventional polymers by complex polymers which can not be obtained by traditional techniques of chemical synthesis¹, ². The usual materials have exhausted their properties in biomedicine and their uses in physical-chemistry treatments³. This point has permitted to focus them as a potential materials to be used in nanotechnology science. This new science combines the materials science with other disciplines that can offer innovative solutions to the current problems. The design of new synthetic devices with enhanced stimuli-responsive sensitivity and targeting ligands is a promising field for the development of cancer-specific delivery systems⁴-⁹. When ionizable groups are incorporated within the polymer network of hydrogels, these ones could show a selective swelling in response to external pH changes¹⁰-¹³. Hydrogels that are sensitive to pH are obtained by incorporating ionizable functional groups into the polymer network¹⁴, ¹⁵. The physical
dimensions of these pH-sensitive hydrogels results from a balance of the electrostatic interactions of charged polymer chains and network elasticity$^{16}$. The common feature of these materials is to maintain their physiological properties, such as mechanical properties and biocompatibility, during the time period in which they are used$^{18,19}$. After that time, the degradation products generated due to a biodegradative process must be disposed of the body$^{20}$.

Drug release and water loss takes place in the initial stage of gel collapse, followed by a slower release as the drug diffuses from the shrunken and physically compacted gel$^{21-25}$. Although the detailed kinetics of drug release from these systems are complex. The incorporation of poly (N-isopropyl acrylamide) (NIPA) into a cross-linked polymer gel generates a matrix that can exhibit thermally-reversible shrinkage or collapse above the Lower Critical Solution Temperature (LCST) of the homopolymer. Smart polymer hydrogels have the potential to be used in a variety of drug-loading and release formats, and their release characteristics can be tailored to a range of target environments.

One of the most used novel methods to obtain nanoparticles is the microemulsion polymerization. Microemulsion is a clear and stable system$^{27}$. The aqueous phase may contain salts or other compounds that can reduce the diameter of the micelles. In contrast to ordinary emulsions, microemulsions form upon simple mixing of the components and do not require the high shear conditions generally used in the formation of ordinary emulsions$^{28}$. The employ of this process as a polymerization method to obtention of nanohydrogels is a novel resource that exploits the valious characteristics of these systems. Various theories concerning microemulsion formation, stability and phase behavior have been proposed over the years$^{29,30}$. For example, one explanation for their thermodynamic stability is that the oil/water dispersion is stabilized by the surfactant present and their formation involves the elastic properties of the surfactant film at the oil/water interface, which involves as parameters, the curvature and the rigidity of the film$^{31}$.

In this paper we report the synthesis and some possible applications for copolymeric nanohydrogels obtained by inverse microemulsion polymerization of $p$-nitrophenyl acrylate (NPA) with N-isopropylacrylamide (NIPA). Our results indicate that these functionalized smart nanohydrogels could be internalized via folic acid receptors and might be used to transport and selectively deliver active drugs.

2. Materials and Methods

2.1 Materials

$N$-Isopropylacrylamide (NIPA) (Aldrich, 97%) was used as received. 4-Nitrophenyl acrylate (NPA) was synthesized from the precursor $p$-nitrophenol following the method reported by Thamizharasi et al.$^{26}$ The radical initiator 2, 2-azobisisobutyronitrile (AIBN, Merck, > 98%) was recrystallized from methanol prior to use. Aerosol AOT (2-hidroxyethyl sulfo succinate) sodium salt) and dibutyl phosphate (DP) was supplied by Fluka. The crosslinking agent ethylene glycol dimethacrylate (EGDMA, Aldrich, > 98%) was used without further purification. Chloroform (for analysis, > 99%), toluene (extra pure grade, > 99%), diethyl ether (for analysis, > 99.7%) and triethylamine (for synthesis, > 99%), were purchased from Merck. Sodium hydroxide (98%), 4-amino methyl pyridine and 2-amino methyl pyridine were supplied by Aldrich.
2.2 Methods

2.2.1. FTIR studies.
Fourier Transform Infrared spectrophotometer FTIR (Nicolet 6700) was employed to obtain spectra of copolymeric nanohydrogels. The nanohydrogels spectrums were collected using Attenuated Total Reflectance (ATR) Smart Orbit accessory. All the spectra were de average of 100 scans with a resolution of 4 cm\(^{-1}\).

2.2.2. Differential Scanning Calorimetry studies (DSC).
Glass transition temperatures (T\(_{g}\)) were measured using a TA Instruments calorimeter (DSC 2920). Standard indium (156.68\(^{\circ}\)C) and zinc (419.58\(^{\circ}\)C) were used for calibration. The thermal analysis for poly (NPA-co-NIPA) copolymers was performed from 0 to 200\(^{\circ}\)C at a heating rate of 10 degrees/minute and under a nitrogen flow (100 mL/min\(^{-1}\)). T\(_{g}\) was assigned by the mid-point criterion.

2.2.3. Nuclear Magnetic Resonance (\(^{1}\)H-NMR) studies.
The nuclear magnetic resonance spectra of nanohydrogels were obtained using CDCl\(_3\) as a solvent in a Bruker ACE instrument (250 MHz) at 20\(^{\circ}\)C; chemical shifts (\(\delta\)) were measured in ppm relative to chloroform (\(\delta = 7.26\)). The copolymer composition was determined using this technique.

2.2.4. UV-vis spectroscopy studies.
The UV-vis spectroscopy was used to verify the incorporation of pH sensitive groups from the 4AMP and 2AMP into polymer backbone. The UV-vis analysis was performed using a CINTRA 303 spectrophotometer equipped with a peltier power supply thermocell.

2.2.5. Quasielastic Light Scattering measurements (QLS).
The QLS was employed to determine the particle size and the particle size distribution of the nanohydrogels. A light scattering spectrophotometer was used at an angle of 90\(^{\circ}\). Intensity correlation function measurements were carried out using a Brookhaven BI-9000AT 522- channel digital correlator, equipped with a water-cooled Argon-ion laser operated at 514.5 nm as a light source. The dried powder samples were dispersed in water and acetone during 24 hours. All measurements were made at 25\(^{\circ}\)C. The size distributions were obtained by CONTIN analysis.

2.2.6. Scanning Electron Microscopy (SEM) measurements.
The SEM micrographies were collected using a JEOL JSM7000F electron microscopy at 10.0kV, equipped with a Field Emission Gang (FEM). An ultrathin coating of electrically-conducting material such gold, gold/palladium, platinum, tungsten or graphite is deposited by high vacuum evaporation or by low vacuum sputter coating of the sample. For these experiments a gold film was deposited over the sample. Sphericity of the synthesized nanohydrogels was confirmed by this technique.

3. Results and Discussion

3.1. Inverse microemulsion system.
The microemulsion polymerization experiments were prepared using water in oil systems. Before the polymerization was begun, the reaction medium was purged by bubbling purified nitrogen for 20 minutes to eliminate oxygen and during the entire reaction. The reaction was performed at 60\(^{\circ}\)C in a 100 mL reactor equipped with mechanical stirring, reflux condenser and a thermal sensor. The surfactant employed to the formation of inverse micelles was Aerosol AOT and DP was added to the system to decrease the micellar diameter. As the oil soluble initiator was used AIBN (1 to 5 % based on the monomers). Different nanohydrogel samples were obtained varying the amounts of initiator, crosslinking agent and dibutyl phosphate. Total conversion was achieved after 7 hours of reaction. The polymer was purified using selective precipitation with acetone and diethyl ether.
3.2. Post-polymerization reactions.
After the purification process, the nanohydrogel was dispersed in ethyl acetate during two hours in a 100 mL reactor with magnetic stirring and 4AMP or 2AMP was added to the system to induce a nucleophilic substitution reaction in the carbonyl group of the NPA substituent in the nanohydrogel (figure 1), with the consequent releasing of o-nitro phenol to the reaction medium.

3.3. FTIR characterization.
The figure 2 shows an IR spectrum of the nanohydrogels. This spectrum confirms the presence of the most important functional groups of the two monomers (NIPA and NPA) in the copolymer skeleton. Thus, we have bands at 2940 cm\(^{-1}\) and 3350 cm\(^{-1}\) that are due to the vibration of the amide protons NH and C-H bonds from the aromatic ring of the NPA. We also have vibration of a carbonyl group in 1740 cm\(^{-1}\) from the NPA and NIPA. The vibration of the NO\(_2\) group from the NPA at 1350 cm\(^{-1}\). A stretching vibration from group C = C at 1646 cm\(^{-1}\) due to the both monomers: NPA and NIPA, and the aromatic confirmation signals at 720 and 840 cm\(^{-1}\) that are coupled with the bending vibration of the amide group from the NIPA and the presence of ester bonding at 1114 cm\(^{-1}\) from the NPA. Similar spectra were obtained for other samples of nanohydrogels.

3.4. DSC characterization.
The nanohydrogels present a single glass transition temperature located at 99°C, that is located between the glass transition temperatures of the two homopolymers, being 104°C for NIPA and 80°C for PNPA. The initial composition of the nanohydrogels was 95-5% NIPA-NPA. To evaluate glass transition temperatures was used heat flux total curve. This transition is promoted by NIPA and NPA precursors, which are resolved due to the relaxation of the NIPA and NPA domains.

3.5. \(^1\)H NMR characterization
The \(^1\)H NMR spectrum shows that the copolymer contains both the NPA and the NIPA monomers by the chemical shifts located at 7.2 and 3.9 ppm, respectively\(^{39}\). By integrating these peaks the composition obtained indicate that the NIPA and NPA proportion in the polymer backbone is 95.64 - 4.39 mol. %, respectively, which is consistent with the original formulation. Table 1 shows the compositions of several NIPA-NPA nanohydrogels synthesized. A \(^1\)H NMR spectrum of the nanohydrogel is shown in figure 3. The peak located at 0.85 ppm corresponds to the terminal protons of the main chain and appears in this region of the spectrum due to interactions with adjacent groups to each carbon atom, so that these bands suggest the presence of interactions with methylene groups as well as carbonyl groups. These interactions are caused by the methylene groups located in positions α and β. In 1.12 ppm we found the resonance of the isopropyllic protons as well as the signal located at 3.96 ppm\(^{44}\). The NPA protons signal appears between 7 and 7.5 ppm due to the presence of an aromatic ring, and for resonance reasons produces the displacement of the band at this position\(^{44, 45}\).

3.6. UV-Vis characterization
The UV-vis characterization shows that the incorporation of 4AMP and 2AMP functional groups into nanohydrogel was successful through a nucleophilic substitution reaction in the carbonyl carbon from the NPA substitute, releasing o-nitrophenol to the average reaction; this last one produces a yellow coloration to the solution that is characteristic of this compound. Figure 4 shows a spectrum of the crosslinked homopolymer of PNIPA (A) that does not absorb in this region. In other hand we can see the spectrum of the nanohydrogel NIPA-NPA (B) that present an absorption band at 274.3 nm due to the NPA substituent. For the modified nanohydrogel with 4AMP (C) and 2AMP (D) are present bands at 254.6 and 259 nm, respectively, due to the incorporation of 4AMP and 2AMP.

3.7. QLS characterization
3.7.1. Influence of initiator and crosslinking agent concentration
The nanohydrogels of NIPA-NPA were synthesized with different initiator compositions ranged between 1 to 5% respect to the total monomer concentration. Table 2 shows the particle size of nanohydrogels with different amounts of AIBN and the monomeric concentration constant in 95-5% mol of NIPA-NPA, respectively. The nanohydrogels show small particle sizes for all the initiator contents, it shows a remarkable effect of AIBN content in their particle diameter. This dependence between the ratio of free radicals to monomers and particle has been also reported by Gao et al.21

Table 2 also shows the particle size of the nanohydrogels with different crosslinking agent concentration and the variation of the particle size with the addition of DP as an oil-soluble salt. It is very common that the swelling of polymer network decreases as a function of cross linking agent content20, 21. As can be observed, when EGDMA concentration increases the particle diameter increases, as it is usually observed. However, there is a limit initiator concentration in which the particle diameter of the nanohydrogel can not decrease more. The incorporation of an oil-soluble salt results in the reduction of the space inside the micelles limiting the particle size. This effect can be observed in table 2. As can be observed when the concentration of DP increases, the particle size decreases to 28 nm. When the concentration of DP is upper or equal to 5% (referred to the total amount of monomers) the particle size does not exhibit an appreciable change and is keep constant around of this value.

3.7.2. Effect of the substituent in swelling-collapse properties (pH sensitivity)
When specific ionizable groups (like 4AMP and 2AMP in this case) are introduced in the polymer network, the nanohydrogel can exhibit a selective swelling-collapse point as a function of the pH. Figure 5 shows the specific swelling-collapse points achieved with the incorporation of 4AMP (A) and 2AMP (B) into polymer. In case (A) we observe that the specific transition point from the swollen state to the collapsed state occurs at pH = 4.5. However, when 2AMP is the substitute in the polymer chain this transition occurs at pH = 5.4. This behavior is due to the chemical structure of the functional ionizable group that in 2AMP case generates more chemical steric impedance due to the position of the substituent in the pyridine ring.46

3.8. SEM characterization
The micrographs obtained with this technique confirm the light scattering results concerning to the size distribution and the particle size of these particles. One micrography of the NIPA-NPA nanohydrogels is shown in figure 6. Similar electron micrographs were obtained for all the samples of nanohydrogels and confirm the spherical shape of the synthesized polymeric nanoparticles.

4. Conclusions
We have synthesized a new copolymeric nanohydrogel by inverse microemulsion polymerization. The success of the synthesis was confirmed by FTIR, 1H NMR and DSC. The size particle was determined by QELS showing an average value of 33 nm. Post polymerization reactions were performed obtaining modified gels showing a response under specific changes of pH and temperature.

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Figure:

Table 1. Composition of nanohydrogels determined by NMR.

| Sample | % mol NIPA | % mol NPA |
|--------|------------|-----------|
| COP 01 | 94.5       | 5.5       |
| COP 02 | 94.5       | 5.5       |
| COP 03 | 94.3       | 5.7       |
| COP 04 | 94.7       | 5.3       |
| COP 05 | 94.6       | 5.4       |
| COP 06 | 94.7       | 5.3       |
| COP 07 | 94.3       | 5.7       |
| COP 08 | 94.5       | 5.5       |

Table 2. Effect of the addition of initiator amount and DP in particle size.

| Sample | %DP | EGDMA % | AIBN % | Dp (nm) |
|--------|-----|---------|--------|---------|
| COP 01 | 0   | 1       | 1      | 45      |
| COP 02 | 0   | 1       | 2      | 43      |
| COP 03 | 0   | 1       | 3      | 40      |
| COP 04 | 0   | 1       | 4      | 38      |
| COP 05 | 0   | 2       | 1      | 36      |
| COP 06 | 0   | 3       | 1      | 40      |
| COP 07 | 0   | 4       | 1      | 42      |
| COP 08 | 0   | 5       | 1      | 42      |
| COP 09 | 1   | 2       | 4      | 35      |
| COP 10 | 2   | 2       | 4      | 33      |
| COP 11 | 3   | 2       | 4      | 30      |
| COP 12 | 4   | 2       | 4      | 28      |
| COP 13 | 5   | 2       | 4      | 28      |
| COP 14 | 6   | 2       | 4      | 28      |
Figure 1. Post polymerization reactions scheme.

Figure 2. FTIR spectrum of NIPA-NPA nanohydrogels.
**Figure 3.** $^1$H NMR spectrum of nanohydrogels NIPA-co-NPA

**Figure 4.** UV-vis analysis of (A) PNIPA, (B) nanohydrogel NIPA-NPA, (C) modified nanohydrogel with 4AMP and 2AMP (D).
Figure 5. QLS measurements of the modified nanohydrogels with (A) 4AMP and (B) 2AMP.

Figure 6. SEM micrographies of the NIPA-NPA nanohydrogels.