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POLY(N-ISOPROPYLACRYLAMIDE) HYDROGEL INCORPORATING SQUARAINE: SYNTHESIS, DRUG DELIVERY AND PHOTODYNAMIC PROPERTIES

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Abstract

In the present work, we describe the fabrication of a thermosensitive hydrogel. To fabricate the hydrogel (Sq1@PNIPAAm), we opted to use biocompatible poly(N-isopropylacrylamide) (PNIPAM) and squaraine dye (Sq1) as the polymer and the crosslinker, respectively. It is noteworthy that Sq1@PNIPAAm can be loaded with fluorescein, and we evaluated the fluorescein release behavior of Sq1@PNIPAAm hydrogel. We noted that on demand sustainable release of fluorescein was feasible upon gradual heating of Sq1@PNIPAAm hydrogel. Furthermore, Sq1@PNIPAAm hydrogels can be used as photosensitizers pertinent to photodynamic therapy (PDT). Our results show that hydrogel possesses favorable biological safety for use in in vitro anticancer studies. In vitro experiments confirmed that Sq1@PNIPAAm hydrogels could kill over 40% of cancer cells. Overall, we have successfully shown that Sq1@PNIPAAm enabled photodynamic therapy. Moreover, fluorescein loading into Sq1@PNIPAAm was possible, and it could be used to successfully accomplish temperature-controlled on-demand release. Given the abundance of low-cost, commercially accessible monomers available for use in hydrogel synthesis, this method offers access to a wide range of functional hydrogels for use in biomedical applications.
Keywords: Poly(N-isopropylacrylamide), Hydrogel, Drug release, Photodynamic therapy, *In vitro*

SKUARİN İÇEREN POLİ(N-İZOPROPİLAKRİLAMİD) HİDROJEL: SENTEZ, İLAÇ SALIMI VE FOTODİNAMİK ÖZELLİKLER

Özet

Bu çalışmada, termo-duyarlı bir hidrojel sentezi açıklanmaktadır. Hidrojeli (Sq1@PNIPAAm) elde etmek için biyouyumlu poli(N-izopropilakrilamid) (PNIPAM) ve skuarain boyasını (Sq1) sırasıyla polimer ve çapraz bağlayıcı olarak kullanmayı tercih ettik. Sq1@PNIPAAm'ın floresin ile yüklenebileceğini ve floresin salım davranışını inceledik. Sq1@PNIPAAm hidrojelinin kademeli olarak ısıtılmasıyla istenildiğinde sürdürülebilir floresin salımının mümkün olduğunu gözlemledik. Ayrıca, Sq1@PNIPAAm hidrojelleri, fotodinamik tedavide kullanılabilecek fotoduyarlaştırıcı özellik göstermektedir. Sonuçlarımız, hidrojelin in vitro antikanser çalışmalarında kullanım için uygun biyo güvenliğe sahip olduğunu göstermektedir. In vitro deneyler, Sq1@PNIPAAm hidrojellerinin kanser hücrelerinin %40'ından fazlasını öldürebileceğini doğruladı. Genel olarak, Sq1@PNIPAAm'ın fotodinamik tedaviyi mümkün kıldığını başarıyla gösterdik. Dahası, Sq1@PNIPAAm'a ilaç yüklemek mümkündür ve sıcaklık kontrollü ilaç salımı başarıyla gerçekleştirilebilmiştir. Hidrojel sentezi için kullanılabilecek ucuz ve ticari olarak erişilebilir monomerlerin bol miktarda bulunması göz önüne alındığında, bu yöntem biyomedikal uygulamalarda kullanılmak üzere geniş bir yelpazede işlevsel hidrojellere erişim sağlar.
Anahtar Kelimeler: Poli(N-izopropilakrilamid), Hidrojel, İlaç salımı, Fotodinamik tedavi, *In vitro*

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1. Introduction

Photodynamic therapy (PDT) is a clinically authorized, noninvasive therapeutic approach used against a range of common disorders [1–6]. In PDT, a photosensitive substance, also known as a photosensitizer, is excited by the absorption of incident light and then transmits energy (or electrons) to molecular oxygen in the surrounding media. This process produces extremely reactive oxygen species (ROS), such as singlet oxygen, superoxide, or hydroxyl radical, which react promptly with the biological elements of the cells. ROS damage cells, eventually causing cell death. PDT requires oxygen, although photosensitizers and light should not injure healthy tissues [7]. Despite recent progress by optimized photosensitizers [8-10], PDT still has some important

limitations. The photosensitizer is primarily responsible for PDT's effectiveness. Regrettably, hydrophobic compounds such as clinically authorized photosensitizers and numerous others tend to agglomerate under physiological settings. In the aggregated form, the photosensitizers are deactivated, thus PDT can be inhibited. Therefore, it is necessary to develop methods to prevent the aggregation of photosensitizers.

One significant subclass of hydrophilic materials are hydrogels. The hydrophilic polymers can be crosslinked chemically or physically to produce them, which creates a three-dimensional network. [8–11]. Thankfully, structural design has made it feasible to modify the characteristics of hydrogels to suit intended uses. Because of its high absorbency, hydrophilicity, biodegradability, and biocompatibility, hydrogels have become more popular[12]. Drug release [16], wound dressings [17,18], biosensors [19], pharmaceuticals [20], tissue engineering [21], regenerative medicines [22], and diagnostics are just a few of the medical applications in which they offer enormous promise [13,14]. Hydrogels in particular have the potential to be used as drug delivery systems due to their three-dimensional network's ability to contain several medicines or nanomaterials. Moreover, hydrogels that are designed in a stimuli-responsive way will enable prolonged, controlled drug release under specific circumstances. This will prevent rapid loss of the drugs and enhance their therapeutic efficiacy [15–19].

Poly(N-isopropylacrylamide) (PNIPAM) hydrogels are a specific type of hydrogel that has gained significant attention due to their unique temperature-responsive properties [20]. At a lower critical solution temperature (LCST) of around 32°C, PNIPAM undergoes a reversible phase transition. Below this temperature, the hydrogel is highly hydrated and swells, while above the LCST, it undergoes a phase change and collapses, expelling water. This transition is often used in applications such as drug delivery and tissue engineering. Crosslinking methods are employed to stabilize PNIPAM hydrogels and control their mechanical properties. The choice of crosslinker and its concentration can impact the LCST and responsiveness of the hydrogel. Researchers are interested in PNIPAM hydrogels for specific applications and exploring their potential in new fields, such as drug delivery systems [21], responsive biomaterials [22], sensors [23] and smart coatings [24].

Herein we present the fabrication of a thermosensitive hydrogel (**Sq1@PNIPAAm**). We opted to use biocompatible PNIPAAm and a squaraine dye (**Sq1**) as the polymer and the crosslinker to fabricate the thermosensitive hydrogel. Therefore, we have obtained a thermosensitive hydrogel incorporating PNIPAAm and **Sq1**, namely **Sq1@PNIPAAm**. To our delight, **Sq1@PNIPAAm** hydrogels can be used as photosensitizer pertinent for PDT. It is noted that on demand sustainable release of fluorescein is feasible upon gradual heating of **Sq1@PNIPAAm**. Consequently, it was successfully demonstrated that **Sq1@PNIPAAm** enabled photodynamic therapy. Moreover, drug loading into **Sq1@PNIPAAm** was possible, and it could be used to successfully accomplish temperature-controlled on-demand release. Given the abundance of low-cost, commercially accessible monomers available for use in hydrogel synthesis, this method offers access to a wide range of functional hydrogels for use in biomedical applications. We are therefore certain that our method is general and may be expanded to easily obtain a wide variety of polymeric hydrogels.

2. Materials and Methods

2.1. List of materials used

N-isopropylacrylamide (Cas No: 2210-25-5), azobisisobutyronitrile (AIBN, Cas No: 78-67-1), Acetonitrile (Merck) , Fluorescein sodium salt (Cas No: 518-47-8), PBS, Ethanol (Merck), Dulbecco's Modified Eagle (Sigma Aldrich), FBS (Sigma Aldrich), Penicillin/streptomycin (Sigma Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cas No: 298-93-1), DMSO (Cas No: 67-68-5), L-Glutamine (Cas No: 56-85-9, G8540) , RPMI 1640 (Cas No: MDL number: MFCD00217820, R8758), Methylene blue (Cas No: 122965-43-9), DPBF (Cas No: 5471-63-6).

2.2. Sq1@PNIPAAm Synthesis

Sq1@PNIPAAm hydrogels were obtained by polymerization of N-isopropylacrylamide (NIPAAm) and **Sq1** using azobisisobutyronitrile (AIBN) an initiator. In 0.25 ml of acetonitrile, NIPAAm (375 mg, 3.3 mmol, 13 M), Sq1 (10 mg, 0.01 mmol, 0.04 M), and AIBN (7 mg, 0.04 mmol, 0.16 M) were dissolved under a nitrogen environment. The mixture was placed into syringes and left in a 55°C preheated oven for a full day. To get rid of unreacted components, the hydrogels were submerged in acetonitrile.

2.3. Gel fraction and swelling

Cylindrical hydrogel samples (4.7 mm in diameter, 2 cm in length) were immersed in excess water at 25°C for 24 hours, with the water being replaced frequently. Weighing the samples allowed us to track the mass m of the hydrogel as a function of swelling time. The gel relative weight swelling ratio m_{rel} was determined as $m_{rel} = m/m_0$, where m_0 is the initial mass of the gel sample. The equilibrium swelling gel samples were then removed from the water and freeze dried. [11]From the masses of the dried, extracted polymer network, the gel fraction Wg (mass of water-insoluble polymer / starting mass of the monomer) was calculated to be 0.58±0.036

2.4. Fluorescein Loading and release with Sq1@PNIPAAm hydrogel

We used fluorescein sodium salt in drug loading and release studies of hydrogels. Drug loading was carried out via the postsynthetic adsorption method. The hydrogels (100 mg) were immersed in fluorescein solution ($1x10^{-3}$ M, 20 ml) in water until complete swelling and saturation for 24 h while stirring (300 rpm) at room temperature. Then, fluorescein-loaded hydrogels were taken from the solution by centrifugation at 5000 rpm for 5 min and washed with water. The amount of fluorescein loaded into hydrogels was calculated from the difference between initial and final concentrations of fluorescein solutions by means of calibration curves at 485 nm. Loading content (LD%) and encapsulation efficiency (EE%) were calculated from the following equations, respectively [25,26]

LD% = (weight of the fluorescein loaded into hydrogels/weight of the hydrogels) x 100

EE% = (weight of the fluorescein loaded into hydrogels/weight of the fluorescein in the initial feed solution) x 100

Drug release experiments were carried out in PBS (pH 7.4) solutions with UV-Vis equipped with a single cell peltier accessory. The release of hydrogels has been investigated through a gradual temperature increase from 28°C to 37°C and

then to 50°C. The percentage of fluorescein released from hydrogels was calculated from the difference between initial and final concentrations of fluorescein solutions by means of calibration curves at 485 nm. The percentage of fluorescein loaded into hydrogels was assumed to be 100%.

2.5. Biocompatibility test

First, hydrogels were immersed in 75% ethanol solution for sterilization. They were washed with Dulbecco's Modified Eagle (DMEM) several times to remove residual ethanol before incubation overnight [18, 27]. DMEM (100 µl) containing 10% FBS and 1% penicillin/streptomycin was added to 96-well plates, and 25x10^3 cells per well were cultured at 37°C by incubating in a 5% CO2 environment overnight in L929 cell lines (ATCC® CRL-6364™). The cells were washed with PBS, and sterilized hydrogels (2.5, 5.0 and 7.5 mg) in DMEM were added to each well before incubation for 24 hours. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cell viability. MTT (11 µl, 5 mg/mL) was added to each well before incubation for 3 hours. Then DMEM was removed and DMSO (100 µL) was added to dissolve the formazan crystals before the absorbance was measured at 630 nm in a microplate reader (ChroMate® 4300 ELISA Reader). The following equation was used to calculate the percentile of cell viability [28].

$$Rv = ((A_1 - A_0) / (A_2 - A_0)) \times 100\ \%$$

where Rv is cell viability, and A_0, A_1 and A_2 are the absorption of blank, sample, and control groups, respectively.

2.6. Cell viability assay

SK-MEL 30/an1 cells were cultured in a regular growth medium consisting of RPMI 1640 with L-Glutamine supplemented with 10% fetal bovine serum under an atmosphere of 5% CO2 at 37°C. **Sq1@PNIPAAm** hydrogels were added to culture media in a 96-well plate. Then the plate was irradiated with red light for 30 min. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A solution of MTT in distilled water (11 µl, 5 mg/ml) was added to each well, followed by incubation for 4 h under the same conditions at 37 °C. Then the medium was discarded and DMSO (100 µl) was added. At ambient temperature, the plate was agitated on a ChroMate® microplate reader before the absorbance at 490 nm was measured. The cell viability was then determined by the following equation: viability (%) = $\{\sum[(A_i / A_{control}) \times 100]\}/n$, where A_i is the absorbance of the corresponding data (i = 1, 2, 3, ..., n), A control is the average absorbance in control wells where the samples were absent, and n is the data points [28].

2.7. Singlet Oxygen Generation with Sq1@PNIPAAm Hydrogel

Methylene blue (MB) was used as a standard for singlet oxygen quantum yield (Φ_Δ) calculations. The hydrogel solution containing DPBF was left in the dark for 20 min before irradiating with red light. Changes in the absorption spectrum of DPBF (410 nm) were recorded. The Φ_Δ values were calculated using the following equation with the method based on the literature [19, 29]

$$\eta_{\Delta Hydrogel} = \Phi_{MB} (S_{MB})/S_{Hydrogel}$$

Here, standard and sample are denoted by the subscripts MB and hydrogel, respectively. $\Phi_{\Delta(MB)}$ is the singlet oxygen quantum yield of MB reported as 0.52 in water [30] S is the slope of the bleaching of the DPBF (410 nm) with respect to the irradiation time.

2.8. FTIR Analysis of Sq1@PNIPAAm Hydrogel

The functional groups in the chemical structure of the hydrogels were analyzed by FTIR spectra using the ID5 ATR of apparatus Thermoscientific Nicolet IS5 in the range of 600 to 4000 cm^{-1}. The spectra were processed using Omnic software.

2.9. TGA Analysis of Sq1@PNIPAAm Hydrogel

To examine the temperature-dependent mass change of Sq1@PNIPAAm hydrogel and determine its thermal stability, a Hitachi Exstar SII TG/DTA 7300 model TGA device was used. The thermal response of Sq1@PNIPAAm was investigated in the temperature range of 25-525 °C with a heating rate of 5 °C/min. Weight loss was recorded in an open pan under an inert nitrogen atmosphere.

2.10. DSC Analysis of Sq1@PNIPAAm Hydrogel

To investigate the phase transition of Sq1@PNIPAAm hydrogel, a Mettler Toledo / DSC 1 / 700DSC model DSC device was used. For this process, Sq1@PNIPAAm was swollen in water until it reached its maximum volume. The glass transition property of Sq1@PNIPAAm was examined in the range of 0°C to 100°C at a heating rate of 1°C/min under an argon atmosphere.

2.11. SEM Analysis of Sq1@PNIPAAm Hydrogel

The morphology of Sq1@PNIPAAm was examined using a FEI Quanta FEG 250 model SEM. Prior to observation, the hydrogels were freeze-dried using a lyophilizer at -80°C for one day and then subjected to vacuum dehydration.

2.12. Statistical Analysis

Data analysis was done with GraphPad Prism 8.0 software. The results of the tests were given as mean ± standard error using a two-way ANOVA, followed by analysis with the Bonferroni posttest. ****P<0,0001 (n=3). Data are expressed as the means of three independent experiments.

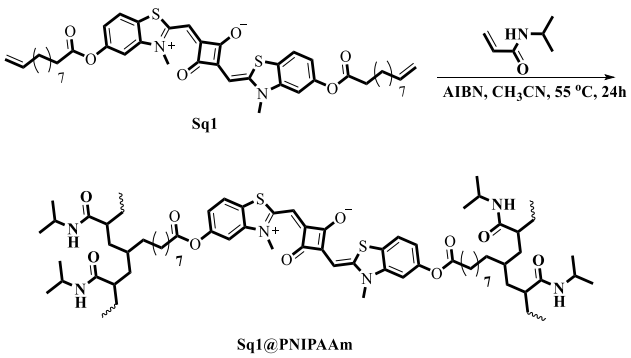
3. Results and Discussion

3.1. Synthesis of Sq1@PNIPAAm hydrogel

Sq1@PNIPAAm hydrogels contain squaraine dye (**Sq1**) as the chemical crosslinker. In the far-red region of the electromagnetic spectrum, where tissue light penetration is comparatively higher than in the visible range, squaraines both emit and absorb light [41, 42].

These characteristics make squaraines attractive to numerous applications, including sensors [43], solar cells [44, 45], and photoinitiators [31]. Unfortunately, the majority of squaraines are hydrophobic and insoluble in water due to their planar structure. They tend to aggregate in aqueous solutions, limiting their use in biomedical applications. Furthermore, physiological instability renders squaraines ineffective in medicinal applications. Therefore, the development of novel techniques is important to extend the overall value of squaraines, particularly in biomedical applications [32]. In this context, we envisaged that incorporation of squaraine as a crosslinker in the hydrogel network will come with extra advantages. First of all, the hydrogel network can act as a permanent shield and maintain the stability of **Sq1** in physiological conditions by keeping it from environmental effects. More importantly, **Sq1** can be used in an aqueous solution without compromising desirable characteristics for

biomedical applications after integration into a hydrogel network, avoiding solubility and aggregation issues despite its hydrophobic nature. Therefore, **Sq1** was chosen as a crosslinker due to its high molar absorption coefficient [33–35]. The synthesis and characterization of **Sq1** were carried out according to literature [36]. We stipulated that **Sq1** could be used as a cross-linker to get **Sq1@PNIPAAm**. Moreover, if the hydrogel could produce ROS upon illumination, this would open the door for **Sq1@PNIPAAm**'s biological applications. For this reason, N-isopropylacrylamide was polymerized at 55°C with **Sq1** and AIBN acting as the crosslinker and initiator, respectively (Scheme 1). **Sq1@PNIPAAm** hydrogel was obtained after 24 h.



Scheme 1. Synthesis of **Sq1@PNIPAAm** hydrogel.

Sq1@PNIPAAm was characterized with scanning electron microscopy (SEM), Fourier transform infra-red spectroscopy (FTIR), thermal gravimetric analysis (TGA), and swelling analysis. SEM images were depicted in Figure 1a. **Sq1@PNIPAAm** had porous structure. The elemental composition of the hydrogel was determined by energy dispersive x-ray spectroscopy (EDX). Figure 1b exhibits three peaks related to carbon, oxygen, and nitrogen elements. The presence of sulfur in the hydrogel network was also confirmed by EDX analysis (Figure 1b). As a result, **Sq1** was incorporated into the hydrogel network since it was the sole structure with sulfur. Therefore, the successful preparation of **Sq1@PNIPAAm** hydrogel was evident. The FTIR spectrum of **Sq1@PNIPAAm** showed the presence of -NH, CH, C=O, C-N and C-O peaks at 3280 cm^{-1}, 2970 cm^{-1}, 1650 cm^{-1}, 1540 cm^{-1} and 1360 cm^{-1}, respectively (Figure 1c). Thermal gravimetric analysis was used to examine the thermal behavior of the **Sq1@PNIPAAm** hydrogel. The **Sq1@PNIPAAm** lost 7% of its mass between 20-100 °C (Figure 1d). This was most likely related to water removal. Then, 78% weight losses were reported between 100-500 °C, which was related to the depolymerization of the hydrogel structure [37–41].

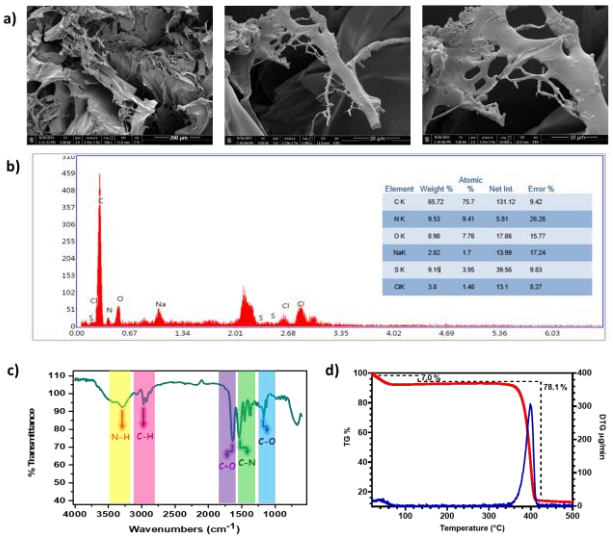


Figure 1. a) SEM images (from left to right; 500x, 5000x and 10000x), the scale bars are 200 μm, 20 μm and 10 μm respectively, b) EDX, c) FTIR spectrum, and d) TGA spectrum of **Sq1@PNIPAAm** hydrogel.

Hydrogels can swell in response to changes in environmental conditions such as pH, temperature, and ionic strength. This property makes them responsive and suitable for controlled drug release and sensor studies in various applications. When placed in an aqueous environment, hydrogels swell as they absorb water, resulting in an increase in volume. The extent of swelling is determined by the chemical composition and crosslinking density of the hydrogel. In an aqueous environment, the **Sq1@PNIPAAm**'s swelling capability was examined. Hydrogels were kept in excess water at 25°C for one day. Swelling of **Sq1@PNIPAAm** hydrogel took around 60 h (Figure 2a). The hydrogels' mass (m) was determined as a function of time. The hydrogels' mass (m) was calculated as a function of time. **Sq1@PNIPAAm** hydrogel had a relative weight swelling ratio (mrel) of roughly 10.67. The vial inversion test, which shows the formation of hydrogel, is depicted in Figure 2b.

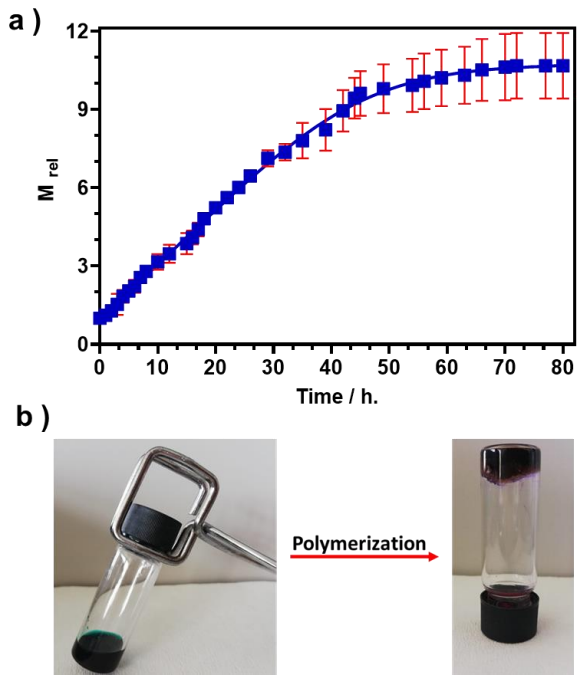


Figure 2. a) Swelling capacity of the **Sq1@PNIPAAm**. b) The digital camera images of vial inversion test for **Sq1@PNIPAAm**.

3.2. Photodynamic properties of Sq1@PNIPAAm

Photodynamic features of the **Sq1@PNIPAAm** hydrogel were tested with chemical scavengers. 1,3-diphenylisobenzofurane (DPBF) is a conventional scavenger molecule used for the detection of $^1O_2$ in situ. As soon as $^1O_2$ is generated by **Sq1@PNIPAAm** hydrogel, it will react with DPBF, and the optical density of DPBF will be diminished upon illumination. Therefore, the photodynamic capacity of **Sq1@PNIPAAm** hydrogel was evaluated with DPBF in PBS solution (pH 7.4). Figure 3a shows the UV-Vis absorption spectral changes of DPBF in the presence of **Sq1@PNIPAAm** hydrogel during illumination. The results clearly indicated that **Sq1@PNIPAAm** hydrogel was photodynamically active and generated singlet oxygen when illuminated with red light. It is noteworthy that the optical density of DPBF is decreased in a time-dependent manner during illumination (Figure 3b). Furthermore, photodynamic tests of **Sq1@PNIPAAm** hydrogel were repeated with p-nitrosodimethylaniline (RNO), which served as a selective singlet oxygen scavenger. It is reported that photosensitizers could induce bleaching of RNO, which can be observed spectrophotometrically [42]. However, it should be noted that RNO is more sensitive and selective for singlet oxygen in aqueous solution when used with imidazole or histidine. Henceforth, we carried out singlet oxygen generation tests with **Sq1@PNIPAAm** hydrogel, RNO, and imidazole in PBS solution (pH 7.4). Delightfully, we observed the bleaching of RNO when light illumination was introduced (Figure 3c). As per our expectation, absorbance of RNO is declined by time during the illumination (Figure 3d). Obviously, the data proved that **Sq1@PNIPAAm** hydrogel could generate singlet oxygen and is effective as photosensitizer. Based on the foregoing experimental data, singlet oxygen quantum yield (Φ_Δ) was determined as 0.02 (the standard was methylene blue with Φ_Δ of 0.52) [28].

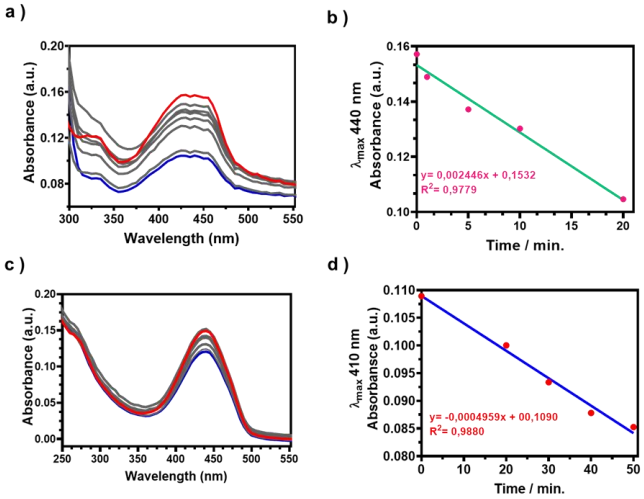


Figure 3. a) UV-Vis absorption spectral changes of DPBF (6.6x10-6 M, λ_{max}: 410 nm) in the presence of **Sq1@PNIPAAm** hydrogel during illumination with red light in PBS (pH 7.4) solution, b) Time-dependent bleaching of DPBF absorption @410 nm in the presence of **Sq1@PNIPAAm** hydrogel during illumination with red light in PBS (pH 7.4). c) UV-Vis absorption spectral changes of RNO ($4.5x10^{-5}$ M)-imidazole ($6.4x\ 10^{-3}$ M) in the presence of **Sq1@PNIPAAm** hydrogel during illumination with red light in PBS (pH 7.4) solution, d) Time-dependent bleaching of RNO absorption @440 nm in the presence of **Sq1@PNIPAAm** hydrogel during illumination with red light in PBS (pH 7.4).

3.3. The lower critical solution temperature (LCST) and drug release features of Sq1@PNIPAAm hydrogel

Poly(N-isopropylacrylamide) (PNIPAAm) is a temperature responsive polymer. The lower critical solution temperature (LCST) of poly(N-isopropylacrylamide) (PNIPAAm) refers to the temperature at which this polymer undergoes a phase transition in solution. LCST of PNIPAAm is typically around 32°C in water. PNIPAAm is hydrophilic below its LCST and becomes hydrophobic above this temperature. Below the LCST (around 32°C), PNIPAM chains interact favorably with water due to hydrogen bonding, leading to a swollen and expanded coil-like structure, allowing water molecules to penetrate. However, as the temperature increases above the LCST, the polymer collapses and becomes more hydrophobic, causing the expulsion of water molecules as it forms a more compact structure, akin to a collapsed coil or globule.

N-isopropylacrylamide was polymerized with **Sq1** to obtain **Sq1@PNIPAAm** hydrogels. Altering the composition of the polymer could change the LCST. Incorporating different monomers or adjusting their ratios could influence the temperature responsiveness of the polymer. The exact LCST can vary depending on factors such as molecular weight, polymer concentration, pH and the presence of additives in the solution. Therefore, we determined the LCST of **Sq1@PNIPAAm** hydrogel. LCST can be determined using differential scanning calorimetry (DSC) [19, 43]. Figure 4a shows the DSC results of **Sq1@PNIPAAm** hydrogel. LCST was found to be 33.95 °C for **Sq1@PNIPAAm** hydrogel. Below this temperature, **Sq1@PNIPAAm** hydrogel was hydrophilic, forming a swollen coil-like structure that interacts favorably with water. As the temperature rises above the LCST (from 28 °C to 57 °C), **Sq1@PNIPAAm** hydrogel underwent a phase

transition, becoming hydrophobic and collapsing into a more compact form, expelling water molecules (Figure 4b).

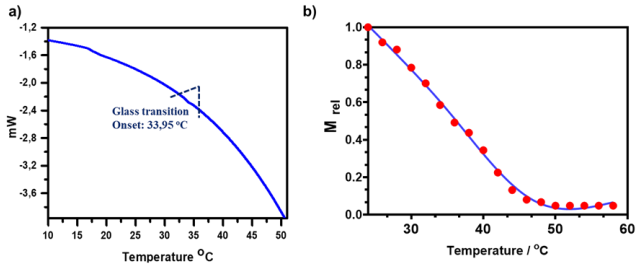


Figure 4. a) DSC of **Sq1@PNIPAAm** hydrogel, b) The deswelling ratio of **Sq1@PNIPAAm** hydrogel in DI water at different temperatures. Inset shows the **Sq1@PNIPAAm** hydrogel before and after heating above LCST.

LCST behavior of **Sq1@PNIPAAm** hydrogel can find applications in various fields, including drug delivery, tissue engineering, and responsive materials, due to its reversible phase transition with temperature changes in aqueous environments. Among these, drug delivery is a promising and versatile approach that offers several advantages, including controlled release of therapeutic agents, improved bioavailability, reduced side effects, and targeted delivery. Hydrogels can be designed to release drugs or therapeutic agents slowly and steadily over an extended period. This sustained release is especially valuable for drugs that require continuous or long-term administration. Controlled drug release from hydrogels can help reduce the side effects associated with some drugs. Hydrogel-based drug delivery systems have been used for a wide range of therapeutic applications, including cancer treatment, pain management, wound healing, and regenerative medicine. Ongoing research in this field continues to explore new hydrogel formulations and delivery strategies to improve drug delivery efficiency and effectiveness. Keeping these facts in mind, we envisaged that **Sq1@PNIPAAm** hydrogel could be loaded with drugs and used as a drug delivery system. The release of drugs can be controlled by adjusting the hydrogel's swelling behavior through temperature changes [36]. To this purpose, we opted to use fluorescein and investigated the premise of **Sq1@PNIPAAm** hydrogel as drug delivery system. The drug loading was achieved by the physical adsorption method. The loading content (LD%) and encapsulation efficiency (EE%) of fluorescein for **Sq1@PNIPAAm** were found to be 0.71% and 23.42%, respectively. Fluorescein-release profile of **Sq1@PNIPAAm** was investigated at different temperatures. The results were depicted in Figure 5. Remarkably, **Sq1@PNIPAAm** hydrogel exhibited temperature-dependent drug release behavior. It was noted that fluorescein release was very slow at ambient temperature and reached around 10.30% (Figure 5, red circles). However, 20.39% of fluorescein was released from **Sq1@PNIPAAm** hydrogel when the temperature was adjusted to body temperature (37°C, Figure 5, blue squares). Finally, **Sq1@PNIPAAm** hydrogel released around 29.24% of fluorescein when the temperature was gradually increased from 28°C to 50°C (Figure 5, green triangles).

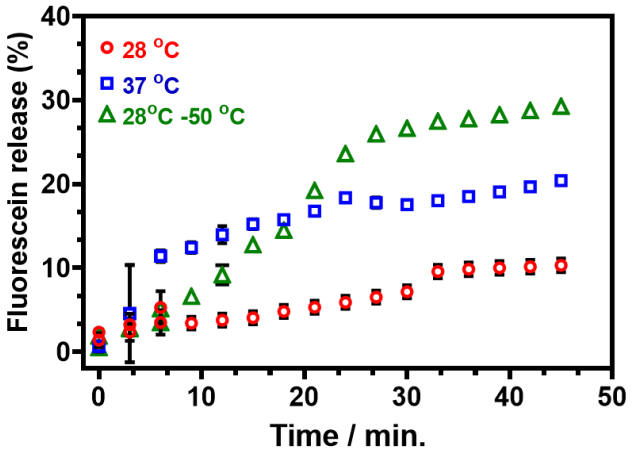


Figure 5. Fluorescein release performance of **Sq1@PNIPAAm** at different temperatures.

3.4. Biocompatibility and in vitro PDT studies with Sq1@PNIPAAm hydrogel

PNIPAAm hydrogels are generally considered biocompatible, which makes them suitable for many biomedical applications. However, it is essential to ensure that the crosslinking agents and any residual monomers are not toxic. The biocompatibility of **Sq1@PNIPAAm** hydrogels was investigated utilizing the L929 fibroblast cell line. Various amounts of **Sq1@PNIPAAm** hydrogel (0.0-6.0 mg) were incubated with the L929 cell line to test the toxicity using an MTT assay. The **Sq1@PNIPAAm** hydrogel was found to be non-toxic to L929 cells (Figure 6a). The cell viability was still quite high (≥80%) even after incubation with 6 mg of **Sq1@PNIPAAm**.

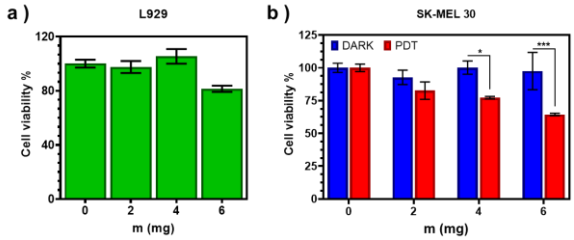


Figure 6. a) Cell viability for L929 cells after incubation with **Sq1@PNIPAAm** hydrogels at various concentrations (0.0-6.0 mg). b) Cell viability for SK-MEL 30 cells after incubation with **Sq1@PNIPAAm** hydrogels at various concentrations (0.0-6.0 mg) with or without illumination (dark) with red light.

The favorable biological safety of **Sq1@PNIPAAm** hydrogel encouraged us to use it in in vitro PDT studies. Therefore, we investigated the photocytotoxicity of **Sq1@PNIPAAm**. Human melanoma cells (SK-MEL 30/An1) were treated with various amounts of **Sq1@PNIPAAm** (0.0-6.0 mg) for this purpose. Hydrogel samples were irradiated with red light for 30 min, whereas control groups were kept in the dark. The MTT assay was used to determine the cell viability. The data of PDT tests with **Sq1@PNIPAAm** are shown in Figure 6b. In the control experiments, where the irradiation step was excluded (dark control), SK-MEL 30 cells showed no appreciable change in cell viability (blue bars in Figure 6b). In stark contrast, the cell viability was decreased when the samples were illuminated with red light (red bars in Figure 6b). In PDT investigations, an effective dose-dependent anticancer effect was observed, and

the inhibition rate was steadily increased up to 40% when 6.0 mg of **Sq1@PNIPAAm** hydrogel was used.

4. Conclusion

Cross-linker engineering was used to create multifunctional polymeric hydrogels. Most importantly, we successfully synthesized PNIPAAm hydrogels with **Sq1** as a covalent cross-linker (**Sq1@PNIPAAm**). We also demonstrated that cross-linker-engineered PNIPAAm hydrogels were efficient photosensitizers for PDT. As an aside, **Sq1@PNIPAAm** hydrogel was tested as a drug carrier. The drug release behavior of **Sq1@PNIPAAm** was studied using fluorescein as a model drug. **Sq1@PNIPAAm** hydrogel allowed the sustainable release of fluorescein upon heating. Hydrogel possesses a favorable biological safety for use in *in vitro* anticancer studies. In vitro experiments confirmed that **Sq1@PNIPAAm** hydrogels could kill over 40% of cancer cells. Exhaustively, it was effectively shown that cross-linker engineering of PNIPAAm hydrogel permitted in vitro photodynamic therapy. Hydrogel network could be loaded with drugs, and temperature-controlled drug release was feasible with the **Sq1@PNIPAAm** hydrogel. Given the number of low-cost and readily available monomers that can be used in hydrogel synthesis, our method provides access to a diverse spectrum of functional hydrogels for biomedical applications. As a result, we are convinced that our approach is unique and can be easily adapted to produce a wide range of polymeric hydrogel-based therapies.

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6. Conflict of interest

The hydrogels of the kind described has been patented through Turkish Patent and Trademark Office (Patent No: 2021/005179) with the permission of Aksaray University.

7. References

[1] Simões JCS, Sarpaki S, Papadimitroulas P, Therrien B, Loudos G. Conjugated photosensitizers for imaging and PDT in cancer research. J Med Chem 2020;63:14119–50.
[2] Shi H, Sadler PJ. How promising is phototherapy for cancer? Br J Cancer 2020;123:871–3.
[3] Escudero A, Carrillo-Carrión C, Castillejos MC, Romero-Ben E, Rosales-Barrios C, Khiar N. Photodynamic therapy: photosensitizers and nanostructures. Mater Chem Front 2021;5:3788–812.
[4] Beharry AA. Next-generation photodynamic therapy: new probes for cancer imaging and treatment. Biochemistry 2018;57:173–4.
[5] Callaghan S, Senge MO. The good, the bad, and the ugly–controlling singlet oxygen through design of photosensitizers and delivery systems for photodynamic therapy. Photochemical & Photobiological Sciences 2018;17:1490–514.
[6] Zhao X, Liu J, Fan J, Chao H, Peng X. Recent progress in photosensitizers for overcoming the challenges of photodynamic therapy: from molecular design to application. Chem Soc Rev 2021;50:4185–219.
[7] Pamuk Algi M, Sarıgöl R. Squaraine-containing polymeric nanoparticles enable photodynamic and photothermal therapy of cancer cells. Dyes and Pigments 2024:112138.
[8] Tuncaboylu DC, Argun A, Algi MP, Okay O. Autonomic self-healing in covalently crosslinked hydrogels containing hydrophobic domains. Polymer (Guildf) 2013;54:6381–8.
[9] Chambre L, Saw WS, Ekineker G, Kiew LV, Chong WY, Lee HB, et al. Surfactant-free direct access to porphyrin-cross-linked nanogels for photodynamic and photothermal therapy. Bioconjug Chem 2018;29:4149–59.
[10] Argun A, Algi MP, Tuncaboylu DC, Okay O. Surfactant-induced healing of tough hydrogels formed via hydrophobic interactions. Colloid Polym Sci 2014;292:511–7.
[11] Algi MP, Okay O. Highly stretchable self-healing poly (N, N-dimethylacrylamide) hydrogels. Eur Polym J 2014;59:113–21.
[12] Bashir S, Hina M, Iqbal J, Rajpar AH, Mujtaba MA, Alghamdi NA, et al. Fundamental concepts of hydrogels: Synthesis, properties, and their applications. Polymers (Basel) 2020;12:2702.
[13] Cha GD, Lee WH, Sunwoo S-H, Kang D, Kang T, Cho KW, et al. Multifunctional injectable hydrogel for in vivo diagnostic and therapeutic applications. ACS Nano 2022;16:554–67.
[14] Li D, Chen K, Tang H, Hu S, Xin L, Jing X, et al. A Logic-Based Diagnostic and Therapeutic Hydrogel with Multistimuli Responsiveness to Orchestrate Diabetic Bone Regeneration. Advanced Materials 2022;34:2108430.
[15] Joo C, Kai D, Lee C-LK. Synergistic antibacterial action of lignin-squaraine hybrid photodynamic therapy: advancing towards effective treatment of antibiotic-resistant bacteria. J Mater Chem B 2023;11:5748–51.
[16] Yao S, Jin X, Wang C, Cao A, Hu J, Chen B, et al. ICG/5-Fu coencapsulated temperature stimulus response nanogel drug delivery platform for chemo-photothermal/photodynamic synergetic therapy. J Biomater Appl 2021;36:565–78.
[17] Meesaragandla B, Sarkar D, Mahalingam V. Methylene blue-loaded upconverting hydrogel nanocomposite: potential material for near-infrared light-triggered photodynamic therapy application. ACS Omega 2019;4:3169–77.
[18] Belali S, Karimi AR, Hadizadeh M. Novel nanostructured smart, photodynamic hydrogels based on poly (N-isopropylacrylamide) bearing porphyrin units in their crosslink chains: A potential sensitizer system in cancer therapy. Polymer (Guildf) 2017;109:93–105.
[19] Belali S, Savoie H, O'Brien JM, Cafolla AA, O'Connell B, Karimi AR, et al. Synthesis and characterization of temperature-sensitive and chemically cross-linked poly (N-isopropylacrylamide)/photosensitizer hydrogels for applications in photodynamic therapy. Biomacromolecules 2018;19:1592–601.
[20] Gulyuz U, Okay O. Self-healing poly (N-isopropylacrylamide) hydrogels. Eur Polym J 2015;72:12–22.
[21] Choi JH, Lee JS, Yang DH, Nah H, Min SJ, Lee SY, et al. Development of a Temperature-Responsive Hydrogel Incorporating PVA into NIPAAm for Controllable Drug Release in Skin Regeneration. ACS Omega 2023;8:44076–85.
[22] Raghuwanshi VS, Joram Mendoza D, Browne C, Ayurini M, Gervinskas G, Hooper JF, et al. Effect of temperature on the conformation and functionality of poly(N-isopropylacrylamide) (PNIPAM)-grafted nanocellulose hydrogels. J Colloid Interface Sci 2023;652:1609–19.
[23] Wang Y, Kan X. LuMA-Functionalized Thermosensitive Hydrogel: A Versatile and Robust Dopamine-Triggered Platform for Diverse Biomolecules Sensing. ACS Appl Bio Mater 2023;6:5097–104.

[24] Maddahzadeh-Darini N, Ghorbanloo M. Supra-Amphiphilic Porphyrin Based on Thermoresponsive Poly (N-Isopropylacrylamide-co-2-Acrylamido-2-Methylpropane Sulfonic Acid Sodium) Hydrogels: Synthesis, Characterization and Catalytic Applications. Catal Letters 2023;153:3342–56.
[25] Toksoy A, Sonkaya Ö, Erkan DS, Gulen RB, Algi MP, Algi F. Norsquaraine endowed with anticancer and antibacterial activities. Photodiagnosis Photodyn Ther 2022;40:103110.
[26] Ayyala RS, S. Suner S, Bhethanabotla VR, Sahiner N. Fungal Keratitis Treatment Using Drug-Loaded Hyaluronic Acid Microgels. ACS Appl Bio Mater 2022;5:3806–15.
[27] Huang K-T, Hsieh P-S, Dai L-G, Huang C-J. Complete zwitterionic double network hydrogels with great toughness and resistance against foreign body reaction and thrombus. J Mater Chem B 2020;8:7390–402.
[28] Degirmenci A, Sonkaya O, Soylukan C, Karaduman T, Algi F. BODIPY and 2, 3-dihydrophthalazine-1, 4-dione conjugates as heavy atom-free chemiluminogenic photosensitizers. ACS Appl Bio Mater 2021;4:5090–8.
[29] Hu QJ, Lu YC, Yang CX, Yan XP. Synthesis of covalently bonded boron-dipyrromethene–diarylethene for building a stable photosensitizer with photo-controlled reversibility. Chemical Communications 2016;52:5470–3.
[30] Usui Y. Determination of quantum yield of singlet oxygen formation by photosensitization. Chem Lett 1973;2:743–4.
[31] Giacoletto N, Ibrahim-Ouali M, Dumur F. Recent advances on squaraine-based photoinitiators of polymerization. Eur Polym J 2021;150:110427.
[32] Yadav Y, Owens E, Nomura S, Fukuda T, Baek Y, Kashiwagi S, et al. Ultrabright and Serum-Stable Squaraine Dyes. J Med Chem 2020;63:9436–45.
[33] Beverina L, Sassi M. Twists and turns around a square: the many faces of squaraine chemistry. Synlett 2014;25:477–90.
[34] Hu L, Yan Z, Xu H. Advances in synthesis and application of near-infrared absorbing squaraine dyes. RSC Adv 2013;3:7667–76.
[35] Khopkar S, Shankarling G. Synthesis, photophysical properties and applications of NIR absorbing unsymmetrical squaraines: A review. Dyes and Pigments 2019;170:107645.
[36] Sarıgöl R, Algi MP. Cross-linker engineering of hydrogel enables photothermal therapy and controlled drug release. J Drug Deliv Sci Technol 2023:104993.
[37] Bhat MA, Rangreez TA, Yaseen Z, Rather RA, Shalla AH. Smart polyacrylamide− cholic acid hybrid composite hydrogel: Development, characterization, and encapsulation study of methylene blue dye. Mater Today Commun 2022;33:104515.
[38] Dave PN, Macwan PM, Kamaliya B. Drug release and thermal properties of magnetic cobalt ferrite (CoFe2O4) nanocomposite hydrogels based on poly (acrylic acid-gN-isopropyl acrylamide) grafted onto gum ghatti. Int J Biol Macromol 2023;224:358–69.
[39] Farahani BV, Ghasemzaheh H, Afraz S. Intelligent semi-IPN chitosan–PEG–PAAm hydrogel for closed-loop insulin delivery and kinetic modeling. RSC Adv 2016;6:26590–8.
[40] Hu X, Feng L, Xie A, Wei W, Wang S, Zhang J, et al. Synthesis and characterization of a novel hydrogel: salecan/polyacrylamide semi-IPN hydrogel with a desirable pore structure. J Mater Chem B 2014;2:3646–58.
[41] Purnomo AS, Bahruji H, Allouss D, El Alaoui-Elbalrhiti I, Subagyo R, Rohmah AA, et al. Hectorite-CTAB–alginate composite beads for water treatment: kinetic, isothermal and thermodynamic studies. RSC Adv 2023;13:790–801.
[42] Kraljić I, Mohsni S El. A new method for the detection of singlet oxygen in aqueous solutions. Photochem Photobiol 1978;28:577–81.
[43] Fu G, Soboyejo WO. Swelling and diffusion characteristics of modified poly (N-isopropylacrylamide) hydrogels. Materials Science and Engineering: C 2010;30:8–13.