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Grafted Hyaluronic Acid Nanogel for the Incorporation of Poly(I:C) as an Immunostimulatory Adjuvant

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ABSTRACT

The aim of this research was to develop a nanogel formulation-based on modified natural polymer, hyaluronic acid (HA), as biodegradable material for adjuvant delivery. Polyinosinic:polycytidylic acid (poly(I:C)) have been approved by FDA as promising adjuvant candidate for the TLR3 (Toll-Like Receptor 3) activation to induce of effective immune system. However, it suffers from being poor stability and is subjected to rapid enzymatic hydrolysis in serum, so that it requires high administered dose leading to the adverse effects. To augment the adjuvant stability and protection from the degradation, the nano-particulate carriers were herein designed with self-assembly of HA scaffold grafted with poly (N-isopropylacrylamide), or pNIPAM. The grafting was processed through amide formation using the coupling agent (EDC/NHS). 1H NMR was carried out to confirm the modified products (HA-g-pNI). The physical incorporation of the nucleic acid into the grafted HA nanogel was achieved by incubation method with the poly(I:C) concentrations of 0.2, 1, and 10 (µg ml-1) in formulations by using 0.1, 0.25, and 0.5 (%w/v) of HA-g-pNI to form the nanogel particles. The mean size, size distribution and surface charge of the nanogel particles were determined by dynamic light scattering (DLS). The particle morphology was investigated by transmission electron microscopy (TEM). Results demonstrated that HA-g-pNI with 4% degree of substitution were formed into nearly spherical nanogel particles with the size of approximately submicron range. The particles presented negative value in zeta-potential showing that poly(I:C) was entrapped. Moreover, we founded that the size and PDI of particles were decreased upon continuous incubation. The development of this poly(I:C)-loaded grafted HA nanogel will lead to the new generation of smart materials that can be functionalized and optimized for different medical purposes.

Keywords: Adjuvant delivery, cancer Immunotherapy, Hyaluronic Acid, nanogels, poly(I:C).

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INTRODUCTION

In recent years, the novel innovation of nanotechnology has been progressing in the field of drug delivery. Among many types of sub-micron particulate drug carriers, the outstanding hydrophilic nanogel-based, non-viral vectors are the systems of interest [1]. Firstly, nanogels can provide special features from their physicochemical properties, compared to other traditional drug delivery systems [2]. Secondly, the nanoscale-sized hydrogels with three-dimensional network structures provide a high water-content property and high biocompatibility that leads to the stability of the colloidal system avoiding particle aggregation in the bloodstream [3]. Thirdly, the tunable polymeric network can be used to incorporate different types of therapeutic molecules including drugs, nucleic acids and proteins [4]. As a result, the payload can be protected from both enzymatic/chemical degradation with the extended the circulation time of the incorporated components [5]. In contrast to the conventional hydrogel or macrogel, the nano-sized hydrogels can be administered through intravenous injection giving the benefit of improved biodistribution. More importantly, hydrophilic nanogels are used to control release of the delivered therapeutic agents by incorporating within polymer networks.

Hyaluronic acid (HA) is a naturally occurring polysaccharide that is mostly present in biological fluids and tissues, especially as a main component of the extracellular matrix and an important molecule for maintenance of cartilage structure [6]. Several research reports indicated that hyaluronic acid-based nanogels have been attractive as a biomaterial used for the development of drug delivery systems. The beneficial properties of these materials over other delivery platforms that are being studied for human clinical trials included 1) the extremely hydrophilic structure of the backbone with high water absorption making them safe for bioorganisms; 2) biodegradability; 3) simplicity for bioconjugation; 4) non-immunogenicity; and 5) high drug loading capacity from the porous polymer networks.

One of the FDA approved potential immunostimulatory adjuvant candidate for novel vaccines is the synthetic double-stranded RNA (dsRNA) that mimics viral RNA, polyinosinic:polycytidylic acid (poly(I:C)). The adjuvant is being used in clinical studies for infectious diseases and cancers. It is recognized by Toll-like receptor 3 (TLR3), which is a transmembrane protein sited on endosomal of antigen-presenting cells (APCs) and many types of tumor cells. Poly(I:C) can encourage the production of the strongest type I interferons and inflammatory cytokines associated with innate and also adaptive immune system [7, 8]. Therefore, poly(I:C) has been promising adjuvants for the development of cancer immunotherapy.

The soluble poly(I:C) possessed some concerns with low efficiency regarding the use in clinic. The molecule is inherently susceptible to rapid enzymatic degradation by RNase in the serum, resulting in a short half-life in human plasma. There are evidences for dose-dependent toxicity after administration of the adjuvant [9, 10]. Currently, besides an immunostimulant poly(I:C) that is widely used

in clinical trials, another strong antiviral adjuvant, poly(ICLC), was designed as an advanced poly(I:C) analog. Poly(ICLC) was developed from complexation of the parent molecule with poly-L-lysine carboxymethyl cellulose. The analog was TLR3 agonist causing innate immune stimulation. It is mostly co-administered with the other immunostimulant vaccines for the maximized therapeutic effects to improve the clinical outcome by delaying the molecular degradation of the original form [11]. However, the of poly(ICLC) efficiency remained similar to poly(I:C) with the comparably low level of cytokine production being elevated [12].

Currently, the major focus in improving bioavailability of the TLR3 agonist, poly(I:C), as a therapeutic vaccine adjuvant includes designing its carrier systems [13]. Efficient delivery system for cancer vaccines are needed to address the instability and systemic toxicity limitations. Various colloidal particulate systems [13-16] have been investigated as carriers for the nucleic acid delivery in order to protect them from enzymatic degradation, elevate their bioavailability, and target the cell or tissue. The expectation lies on promising results in the improvement of the adjuvant activities, which could lead to administered dose reduction and absence in systemic side effects. As a matter of fact, the incorporation of adjuvant and carrier systems are being studied in both non-clinical and clinical trials [17].

In this work, to overcome the limitations of nucleic acid-based immunostimulants, the promising drug delivery systems have been used to improve stability and bioavailability of an immunostimulant poly(I:C). We developed and designed the self-assembly of the hybrid HA backbone and pNIPAM to obtain a biocompatible carrier for encapsulating poly(I:C). Poly(N-isopropylacrylamide) (pNIPAM) was the main feature for the nanogel formation that required grafting on the HA backbone using EDC/NHS coupling chemistry. PNIPAM is one of the most well-known thermal sensitive polymer that made up from hydrophobic (isopropyl group) and hydrophilic (amide group) residues, as it undergoes reversible phase transition when the temperature above its lower critical solution temperature resulting in the conformational and property alterations [18, 19]. In this regard, pNIPAM exhibits a lower critical solution temperature (LCST) of 32ºC, which is a large benefit in many biomedical applications, in particular for drug delivery systems, because it is close to the temperature of the human body [20]. Each concentration of poly(I:C) (0.2, 1, and 10 ($\mu g \cdot mL^{-1}$)) will be loaded into the grafted nanogel with each concentration of the proprietary polymer (0.1, 0.25 and 0.5 (%w/v)) by incubation method. These nanogel formulations were further characterized for the successful self-assemble nanogel formation by DLS, and TEM for their morphology determination. Loading of poly(I:C) was also confirmed.

METHODOLOGY

A. Materials

Sodium hyaluronate (MW 47 kDa, Liuzhou Shengqiang Biotech Co., Ltd, China), poly-(N-isopropylacrylamide) (pNIPAM; MW 5.5 kDa) and N-hydroxylsuccinimide (NHS) (Sigma-Aldrich, USA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (CreoSalus Inc., USA) were used as received. As a TLR3 agonist, poly(I:C) was supported from faculty of medicine, Chulalongkorn university. All chemicals and reagents were purchased from commercial company and used according to manufacturer's instructions.

B. Methods

Synthesis of pNIPAM grafted Hyaluronic Acid.

The nanogel will be prepared by self-assembling of amine-terminated pNIPAM (pNIPAM-NH_2) grafted to hyaluronic acid (HA) backbone via the EDC and NHS peptides coupling chemistry for amide conjugation. Briefly, the conjugating product was achieved by dissolving 47×10^3 ($g\cdot mol^{-1}$) HA in ultrapure water at 0.5% (w/v) and adding the amounts of pNIPAM-NH_2 with M_n equal to 5.5×10^3 ($g\cdot mol^{-1}$) at 0.3465 (g) for grafted HA polymers. Next, EDC/NHS was then added in powder to HA-pNIPAM solution by 1:4 molar ratio of HA to EDC/NHS coupling chemical (0.966 (g) of EDC and 0.58 (g) of NHS) to activate the pNIPAM-NH_2 and HA polymers at room temperature. The pH was adjust to 5.5 and then 7.5 after an hour. The grafting reaction was allowed for 48 hours at room temperature under constant stirring before purifying by dialyzing against ultrapure water to remove unreacted synthesis parent compounds following by the lyophilization 3 days. ^{1}H NMR was carried out to confirm the resulting products.

The synthesized polymer was named HA-g-pNI. The samples in D_2O will be used for ^{1}H NMR spectra and confirm the conjugation of pNIPAM to HA backbone from the integration ratio between their characteristic peaks. The physical characterization (size and zeta-potential) of the nanogel particles was determined by a Zetasizer Nano ZS analyzer (Malvern Instruments Ltd.), and the particles morphology was investigated under a TEM.

Preparation of HA-g-pNI Nanogels and Nucleic Acid Loading.

To prepare poly(I:C) incorporating HA-g-pNIPAM nanogels, gel was prepared by a simple sonication method in aqueous conditions using 0.1, 0.25, and 0.5 (%w/v) of HA-g-pNI polymer in sterile water. After sonication for 30 minutes at room temperature, the nanogels was settled for overnight at 4°C and then centrifugation for 5 minutes at 3000 g. An incubation method will be performed for the HA-g-pNIPAM nanogel loaded with poly(I:C). In brief, 100 µL of poly(I:C) in concentrations of 0.2, 1, and 10 ($\mu g\cdot mL^{-1}$) were then added dropwise to 1 mL of each HA-g-pNI solutions as above. The poly(I:C) incorporated nanogels spontaneously formed with constant stirring at room temperature for 30 minutes, leading to poly(I:C)-loaded HA-g-pNI nanogels. The prepared nanogels were stored at 4°C. These nanogel formulations with the poly(I:C) loads were characterized by using DLS for their diameter size, size distribution, and net surface charge of the particles, and further the TEM image was performed for their morphology.

Statistical Analysis.

Statistical analysis was carried out using IBM SPSS Statistics 23 with one-way ANOVA for characteristics. All studies were performed in triplicate. Results were indicated as a mean and standard deviation of each dataset (mean ± SD), and p values of 0.05 or less was accepted the statistically significant difference.

RESULTS AND DISCUSSION

Synthesis and Characterization of pNIPAM grafted HA

To achieve an effective nucleic acid delivery of the nanocarrier, HA-pNIPAM (can be abbreviated here as HA-g-pNI) grafted copolymer was prepared as previously reported [21, 22]. EDC/NHS coupling reaction was used to

coupling pNIPAM to hyaluronic acid by amide conjugation, as shown in Figure 1. The structure of each conjugating reaction product in polymer synthesis was confirmed via ^{1}H NMR spectra (see figure 1). The pNIPAM-grafted HA was produced with degree of substitution of approximately 4.4%.

Physical Characterization of Adjuvant-free Nanogels

Here, we fabricated the self-assembled HA-g-pNI nanogels by subsequent sonication of the grafted polymer to obtain the nanogel particle formulation. Three different polymer concentrations were used to form the nanogel formulations in water at 0.1, 0.25, and 0.5 (%w/v) of the HA-g-pNI polymer. Characterization for the mean size, PDI, and zeta-potential were investigated by DLS to observed physical properties in each of the formulations.

In this study, we indicated that the concentration of polymer (%w/v) has direct effect to the diameter size of nanogel particle. We found that the particle size of the nanogels increased as the concentration increased (the data was indicated as mean ± standard deviations; table 1). The DLS results showed the fine controlled of particle size of blank nanogels within approximately 700-900 nm of diameter range (see figure 2b). The sizing indicated the different signification in statistical analysis comparing between the nanogel formulations with the grafted polymer at 0.1 and 0.5 (%w/v), and at 0.25 and 0.5 (%w/v). However, the mean particle size difference was not signification, statistically, when comparing nanogel formulations with 0.1 and 0.25 (%w/v) of polymer. The size distribution of nanogel solution was determined as the PDI values. The nanogel solutions showed PDI values of approximately 0.37-0.66, indicating these formulations are quite polydispersed as the characteristic of swellable nanogel (see figure 2b and table 1). The zeta-potential of the HA-g-pNI nanogel formulations displayed small negative charge repulsion in the water of approximately -17 to -26 mV. This probably caused by the remaining carboxylic group on HA parent chain. The small variations in zeta-potential among each formulation was not statistically signification (see figure 2b and table 1).

Preparation and Characterization of the Adjuvant- loaded Hyaluronic Acid Nanogels

Biodegradable nanogels, particularly hyaluronic acid (HA) based nanogel formulations have attracted significant attention as nanomaterials for the design of biotherapeutic delivery carriers [23]. In this study, we successfully fabricated the HA-grafted pNIPAM nanogel carriers, with encapsulation of an TLR3 immunostimulatory adjuvant, poly(I:C) within HA-g-pNI nanogel network (figure 2a). The association of the adjuvant and polymeric assembly was based on a simple entrapment method via physical interaction between polymers and the adjuvant.

The HA-g-pNI nanogels were prepared by grafting pNIPAM to HA, further sonication method for obtaining the self-assembled nanogel particle, and introduction of poly(I:C). 0.1, 0.25 and 0.5 (%w/v) of each polymer in water were carried out to form nanogels formulations by incubation with each of poly(I:C) concentrations. The next day, the physical properties of the adjuvant loaded nanogels were characterized by DLS measurement.

The nucleic acid–polymer ratio enabled control over nanogel size. We observed such effect when keeping the amount of HA-g-pNI constant with varied amount of the loaded adjuvant. The formulations with 0.1% (w/v) HA-g-pNI loaded with poly(I:C) at 0.2 and 1 ($\mu g \cdot mL^{-1}$) resulted in smaller average diameters of the nanogels, with the sizes close to 400 nm with PDI around 0.4, comparing to their blank nanogel without poly(I:C) and the nanogel loaded with 10 $\mu g \cdot mL^{-1}$ poly(I:C) (see figure 2c and table 1).

No significant differences in average sizes (around 200-1,100 nm range; PDI: 0.6-0.7) per poly(I:C) concentration rise in the group of formulations containing 0.25% (w/v) HA-g-pNI (see figure 2d and table 1). The mean particle size of each of 0.5% (w/v) HA-g-pNI formulations with poly(I:C) encapsulation had significant size difference in the range of 300-1,000 nm with the increasing concentration of poly(I:C) loads, while high PDI values varied in the range around 0.6-0.9 (see figure 2e and table 1). The size of 0.5% (w/v) gel loaded with adjuvant concentrations at 0.2 and 1 $\mu g \cdot mL^{-1}$ reduced with significant differences (around 300-600 nm range; PDI: 0.6-0.8) when compared to the blank nanogel. All nanogels presented the negative charge with no significant differences in the range of -14 to -23 mV of the particle surface charges.

Moreover, we observed slight change in size distribution across all nanogel formulations with different incubation time. Both 0.1 and 0.25 (%w/v) HA-g-pNI loading adjuvant concentrations of 0.2 and 1 ($\mu g \cdot mL^{-1}$) had significantly decreased in the average size (by 100-200 nm) upon the continuous incubation for 10 days at 4 ºC compared with day 0 measurement, as in shown figure 2f and table 1. Noticeably, all concentration of poly(I:C) incorporations with 0.5% (w/v) HA-g-pNI polymer showed no differences in particle size alterations per incubation time. No significant differences in the zeta-potential changes in all formulations, accordingly. The study suggested that the longer incubation time could contribute to the smaller size and size distribution of the poly(I:C) nanogels in water.

Morphology of the Nanogel Formulations

The morphology of nanogel particles were indicated by TEM images, shown in figure 3. TEM images represented the HA-g-pNI nanoparticles formed with three different amounts of polymer in aqueous dispersion. They were almost spherical in shape with rough edge, possibly as the particles' swollen surface. Polydispersed size of around 200 nm to 1 µm was demonstrated supporting the hydrodynamic sizing by DLS. The particles were well distributed due to the slight negatively charge on the nanoparticle surface. The nanoparticles formed with poly(I:C) showed even more irregularly spherical shape with higher degree of roughness in their surface nanostructures. Moreover, the size in diameter varied between 200-1,000 nm range, as illustrated in Fig. 3A and 3C.

CONCLUSION

In summary, poly(I:C)-loaded biodegradable nanogels were developed and demonstrated for their physical properties. The goal of this study was the proof-of concept based on the design and development of the nano-delivery systems by grafting the hyaluronic acid backbone with the thermal sensitive polymer, pNIPAM, for advanced application in incorporation with bioactive compounds to enhance their efficiency. However, to improve the advantages of the poly(I:C)-loaded nanogel system for therapeutic use, further study is needed for its biological properties, in vivo, and biocompatibility of the formulations.

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Table 1: Physical properties of the HA-g-pNI nanogels and the poly(I:C) loaded nanogels in each formulation measured by DLS.

Nanogel formulations	Z-average (d.nm)	PDI	Zeta-potential (mV)
I. Blank nanogels			
0.25% HA-g-pNI	754.8 ± 14.71	0.66 ± 0.521	-24.6 ± 3.13
0.5% HA-g-pNI	941.8 ± 69.88	0.37 ± 0.316	-17.5 ± 0.75
II. Nanogels (% w/v) + loaded poly(I:C)(μg·mL^{-1}).			
0.1% + 0.2	389.3 ± 92.71	0.46 ± 0.032	-22 ± 1.7
0.1% + 1	369.1 ± 98.71	0.48 ± 0.047	-14 ± 10.9
0.1% + 10	627.3 ± 92.53	0.56 ± 0.121	-20 ± 6.5
0.25% + 0.2	1095 ± 688.54	0.68 ± 0.226	-23 ± 6.1
0.25% + 1	303.9 ± 289.65	0.86 ± 0.234	-20 ± 6.1
0.25% + 10	493.9 ± 273.54	0.72 ± 0.224	-20 ± 7.6
0.5% + 0.2	313 ± 45.27	0.87 ± 0.148	-23 ± 4.8
0.5% + 1	578 ± 49.75	0.63 ± 0.134	-21 ± 5.0
0.5% + 10	1034.9 ± 88.26	0.59 ± 0.132	-23 ± 5.0
III. Nanogels (% w/v) + loaded poly(I:C)(μg·mL^{-1}): upon incubation continuously at 4ºC.			
0.1% + 0.2	103.2 ± 2.65	0.52 ± 0.525	-19 ± 1.2
0.1% + 1	172 ± 2.41	0.37 ± 0.101	-22 ± 2.4
0.1% + 10	126.8 ± 2.58	0.38 ± 0.105	-21 ± 2.9
0.25% + 0.2	116.6 ± 1.80	0.48 ± 0.004	-25 ± 5.1
0.25% + 1	155.2 + 5.88	0.50 ± 0.013	-24 ± 5.1
0.25% + 10	322.3 ± 28.12	0.45 ± 0.088	-20 ± 5.0
0.5% + 0.2	310.4 ± 10.10	0.38 ±0.132	-22 ± 3.6
0.5% + 1	556.4 ± 88.87	0.55 ± 0.401	-21 ± 5.0
0.5% + 10	1,077.3 ± 143.46	0.47 ± 0.396	-24 ± 3.0

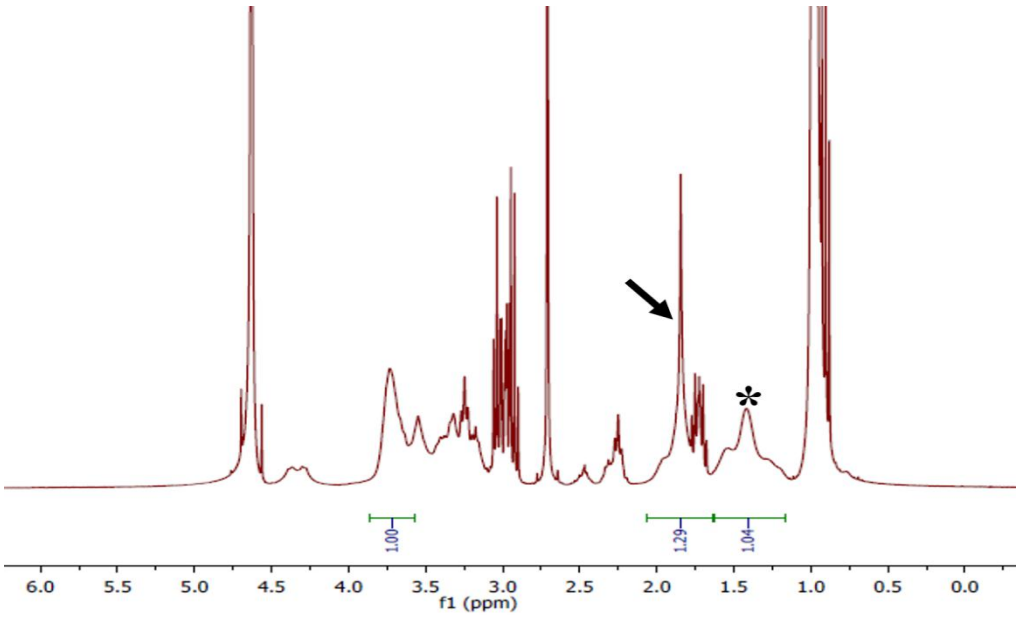

Figure 1: ^{1}H NMR spectra of pNIPAM-grafted hyaluronic acid (HA-g-pNI) with 4% degrees of modification. Arrowed peak indicates the protons that interfere between a proton on a chiral carbon of pNIPAM chain and protons belong to the N-acetyl group of HA backbone. Asterisked peak represents the H_2 protons on C1 at the attached pNIPAM chain.

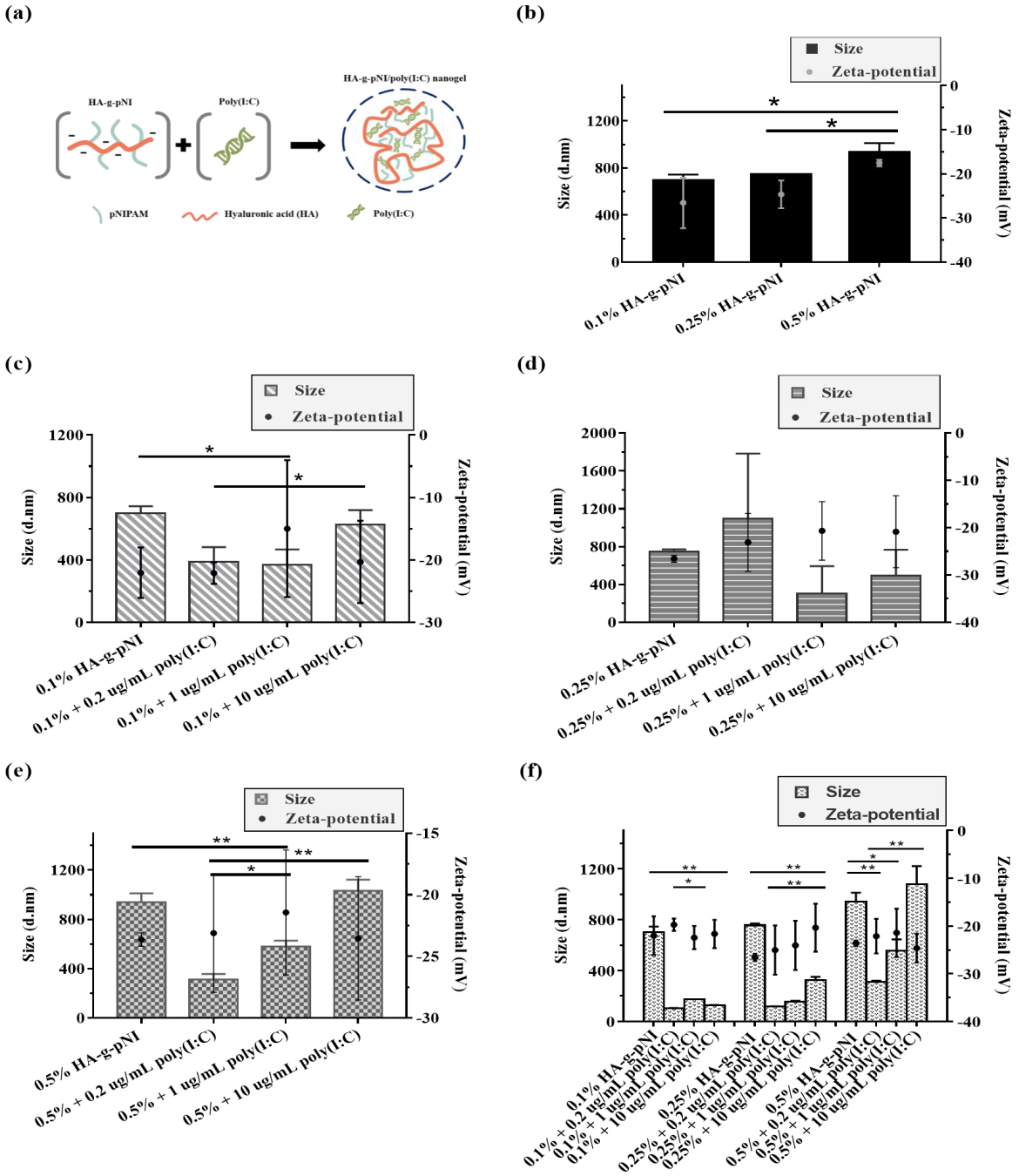

Figure 2: Physical properties of HA-g-pNI nanogels and adjuvant-loaded HA-g-pNI nanogels. The mean size and zeta-potential value of the nanogel particles measured by DLS. (a) Schematic representation of poly(I:C)-loaded HA-g-pNI nanogels prepared by physical incorporation. (b) Size and surface charge of the blank HA-g-pNI nanogels. Comparison between the mean size and surface charge of the blank nanogels and loaded nanogels within each of polymer ratios, including (c) 0.1, (d) 0.25, and (e) 0.5 (%w/v). (f) Size and surface charge of each nanogel formulation when incubation (for 10 days) at 4 ºC. The signification were indicated as *$p<0.05$ and **$p<0.001$.

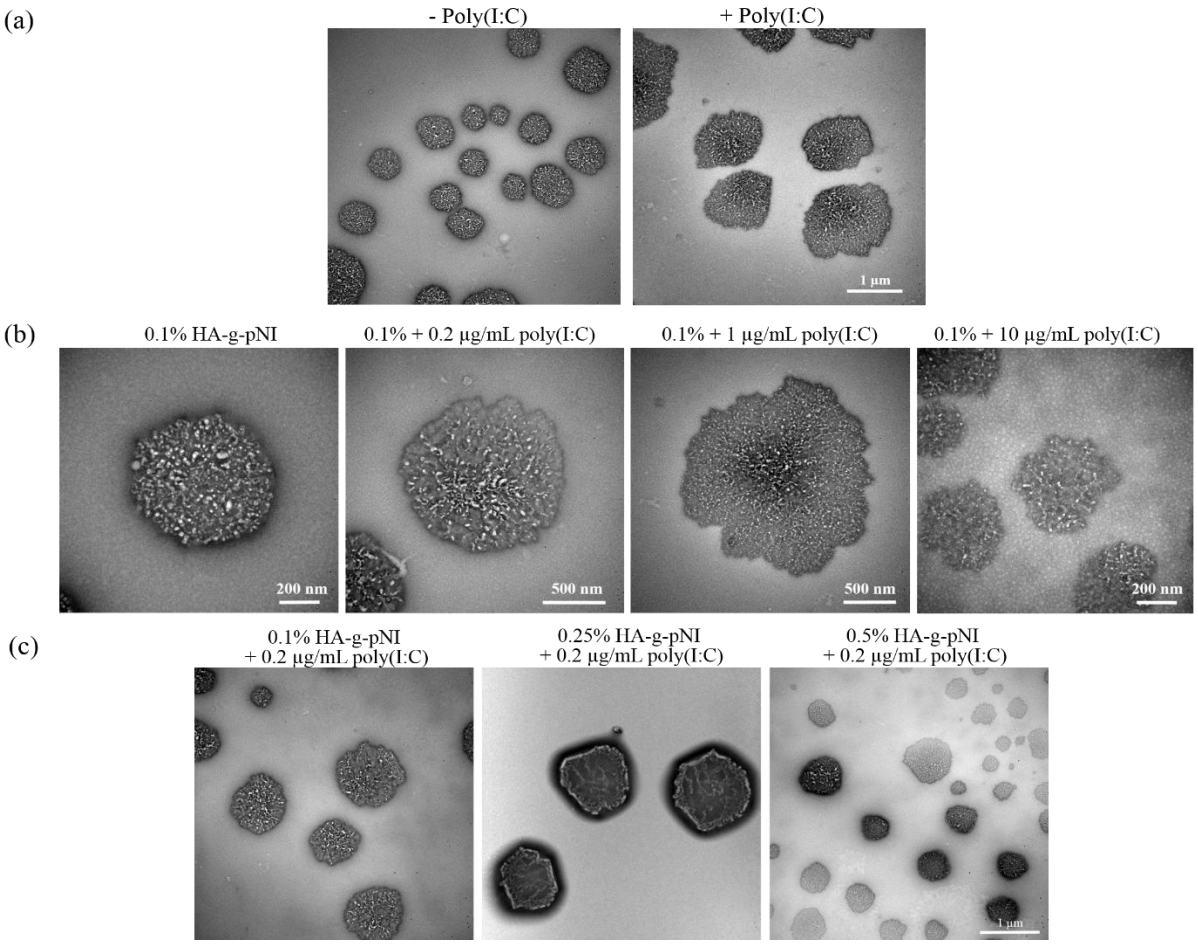

Figure 3: The particle morphology of assembled nanogels with 4.4% degree of grafting measured by TEM. (a) The comparison between the nanogels without the adjuvant and the nanogels incorporating poly(I:C). (b) Each poly(I:C) loaded nanogel formulations resulted from the three different amounts of the poly(I:C) adjuvant. (c) TEM images of the nanogels loaded with the same poly(I:C) amounts in the three different polymer ratios. Scale bar shown 1 µm in parts a and c, and nanoscale level in part b.