

Hyaluronic acid-loaded p(HEMA) nanoparticles reduce the viability of SH-SY5Y neuroblastoma cells in a time-dependent manner

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ABSTRACT

Objectives: This study aims to evaluate the effects of poly(2-hydroxyethyl methacrylate) [p(HEMA)]-based nanoparticles loaded with hyaluronic acid (HA) on the SH-SY5Y human neuroblastoma cell line.

Materials and methods: Three experimental groups were established: free HA, unloaded p(HEMA), and HA-loaded p(HEMA) nanoparticles. Treatments were applied for 12 and 24 h. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, which measures metabolic activity, and the resulting absorbance values were comparatively analyzed.

Results: According to the results, HA-loaded p(HEMA) nanoparticles did not exhibit a negative impact on cell metabolism at 12 h; however, a reduction in viability was observed at 24 h. These findings imply that prolonged release may contribute to cytotoxic effects. In contrast, free HA was found to exert a proliferative effect on the cells. For the unloaded p(HEMA) group, an initial decrease in cell viability was observed, which appeared to diminish over time.

Conclusion: These findings indicate that HA-containing carrier systems may elicit time- and content-dependent cellular responses and hold potential for neurological targeting.

Keywords: Controlled release, cytotoxicity, hyaluronic acid, nanoparticle, p(HEMA), SH-SY5Y.

SH-SY5Y is a human neuroblastoma cell line derived from the embryonic neural crest, widely used as a model in numerous neuroscientific and oncological studies, including neurotoxicity, synaptic transmission, cell differentiation, and drug screening applications. Due to its ability to grow in both adherent and suspension forms, its capacity for differentiation, and its high viability rates, it serves as an ideal cell line for both basic neuroscience research and preliminary evaluations in cancer biology.^[1,2]

This cell line is particularly utilized to evaluate the cytotoxic and proliferative effects

of drugs targeting the central nervous system (CNS), and is frequently preferred for testing carrier systems with enhanced bioavailability. At this point, the blood-brain barrier (BBB) represents one of the major obstacles to effective drug delivery to intracranial tumors. The tight junctions between brain capillary endothelial cells, along with the absence of fenestrations and pinocytosis, significantly limit the passage of therapeutic agents into the CNS. While the BBB serves a physiological function in protecting the brain from toxic substances, its high selectivity poses a challenge for delivering therapeutic molecules to brain tissue.^[3,4]

Nanoparticle-based carrier systems have emerged as promising approaches for targeted drug delivery, enhancement of bioavailability, and reduction of systemic side effects. In particular, hyaluronic acid (HA) is a biocompatible polymer widely used in targeted cancer delivery due to its interaction with

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cluster of differentiation 44 (CD44) receptors. Hyaluronic acid can inhibit cell proliferation and induce apoptosis in certain cancer cell lines through CD44-mediated pathways. Especially high molecular weight HA fragments have been shown to exert growth-inhibitory effects on tumor cells.^[5,6] When combined with structures such as poly(2-hydroxyethyl methacrylate) [p(HEMA)], carrier systems can be engineered to enable controlled release and suitable biodegradability. It is anticipated that the cellular effects of HA may vary over time within such systems, and that the carrier matrix may modulate this effect.^[7,8]

This study aims to evaluate the *in vitro* cytotoxic effects of HA-loaded p(HEMA) nanoparticles on the SH-SY5Y neuroblastoma cell line. Free HA, unloaded p(HEMA), and HA-loaded p(HEMA) were comparatively applied, and their effects on cell viability were analyzed at different time points. Data on the combination of SH-SY5Y cells with HA-loaded p(HEMA) systems are currently scarce in the literature, and this study is expected to contribute novel insights to the field.

MATERIALS AND METHODS

Cell culture

In this study, the SH-SY5Y human neuroblastoma cell line was used as an *in vitro* model to evaluate the cytotoxic effects of HA-loaded pHEMA nanoparticles. The nanoparticles used in this study were provided by Prof. Dr. Sinan Akgöl from Ege University. The SH-SY5Y neuroblastoma cells were kindly provided from Manisa Celal Bayar University. Hyaluronic acid has been widely studied in recent years for its potential role in enhancing targeted delivery via CD44-mediated internalization in neuroblastoma and other tumor models.^[9] However, while cytotoxicity was investigated, apoptotic mechanisms were not within the scope of this study and remain a subject for future research. All experimental procedures were conducted in a cell culture laboratory equipped with appropriate biosafety conditions and in accordance with international standards. The study was conducted in accordance with the principles of the Declaration of Helsinki.

SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, D6429) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, F0804) and L-glutamine (Sigma-Aldrich, G8540), maintained at 37°C in a humidified incubator with 5% CO₂ under standard cell culture conditions. Cells at approximately 70% confluency were used for experiments. Cells were detached from the flask surface and collected by centrifugation at 1000 × g for 2 min. The collected cells were resuspended in fresh medium, and cell density was determined using the trypan blue exclusion assay.

Preparation of nanoparticles and plate seeding

The HA-loaded and unloaded p(HEMA) nanoparticles were prepared under sterile conditions and washed with phosphate-buffered saline prior to experimentation. Subsequently, they were plated into 96-well plates. SH-SY5Y cells were added to each well at the predetermined cell density, and four experimental groups were established: the negative control group, in which cells received no treatment; the first positive control group, treated with free HA (3.5 mg/mL); and the second positive control group, treated with unloaded p(HEMA) nanoparticles, as shown in Table 1. In the experimental group, cells were exposed to HA-loaded p(HEMA) nanoparticles (3.5 mg/mL HA). All groups were incubated for 12 and 24 h, after which cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, as shown in Table 2.

MTS cell viability assay

Cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). In the experiment, 20 µL of MTS reagent was added to each well, and the plates were incubated for two hours at 37°C in a humidified incubator containing 5% CO₂. At the end of the incubation period, absorbance values were measured at 490 nm using a microplate spectrophotometer. The measured absorbance values were used to calculate the percentage of cell viability, as shown in Figure 1.

Table 1. Control and experimental groups

Negative control group	Untreated SH-SY5Y cells
Positive control group 1	SH-SY5Y cells + HA (3.5 mg/mL)
Positive control group 2	SH-SY5Y cells + unloaded p(HEMA)
Experimental group	SH-SY5Y cells + 3.5 mg/mL HA-loaded p(HEMA)

HA: Hyaluronic acid; p(HEMA): Poly(2-hydroxyethyl methacrylate).

Statistical analysis

Statistical analyses were performed manually using statistical formulas within the Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) environment. Data are presented as mean \pm standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

RESULTS

In this study, to evaluate the cytotoxic effects of HA-loaded p(HEMA), SH-SY5Y neuroblastoma cells were treated with p(HEMA), and their effects on cell viability were examined in a time-dependent manner. Experimental assessments were conducted at two different time points, 12 and 24 h, with two replicates performed for each experimental group to ensure result consistency (Table 2, Figure 1a b).

Cell viability measurements were performed using the MTS assay, a metabolic activity-based method. This assay indirectly but reliably assesses cell viability by measuring absorbance

at 490 nm, reflecting metabolic activity. The measurement results demonstrated consistency among technical replicates across time points and allowed the use of mean absorbance values for calculating percentage viability (Figure 1a, b).

At the 12-h time point, the highest mean absorbance values were observed in the "SH-SY5Y Cells + HA-loaded p(HEMA)" group (0.2005) and the "Untreated cells" group (0.1975), (Table 2). These findings indicate that short-term exposure to the HA-loaded p(HEMA)-based nanoparticles does not adversely affect cell viability, with metabolic activity remaining close to that of the control group. The "SH-SY5Y Cells + free HA" group showed a mean absorbance value of 0.1755, suggesting that free HA may exert a neutral or protective effect on cell metabolism in the early phase.

Conversely, in the p(HEMA)-only treatments lacking HA, a significant reduction in metabolic activity was observed, particularly in the "SH-SY5Y Cells + unloaded p(HEMA)" group (0.102), (Table 2). These results suggest that direct interaction of the p(HEMA) matrix with cells may induce potential cytotoxic effects, which can be mitigated by the presence of HA (Figure 1b).

Table 2. Three replicates and the mean absorbance values obtained for each group at 12- and 24-h time points

Experimental group	12-h Replicate 1	12-h Replicate 2	12-h Average	12-h Replicate 1	12-h Replicate 2	12-h Average
Untreated SH-SY5Y cells	0.198	0.197	0.1975	0.324	0.353	0.3385
SH-SY5Y cells + HA (3.5 mg/mL)	0.168	0.183	0.1755	0.376	0.383	0.3795
SH-SY5Y cells + unloaded p(HEMA)	0.113	0.091	0.102	0.251	0.307	0.279
SH-SY5Y cells + 3.5 mg/mL HA-loaded p(HEMA)	0.223	0.178	0.2005	0.201	0.255	0.228
Blank	0.092	0.082	0.087	0.114	0.112	0.113

HA: Hyaluronic acid; p(HEMA): Poly(2-hydroxyethyl methacrylate).

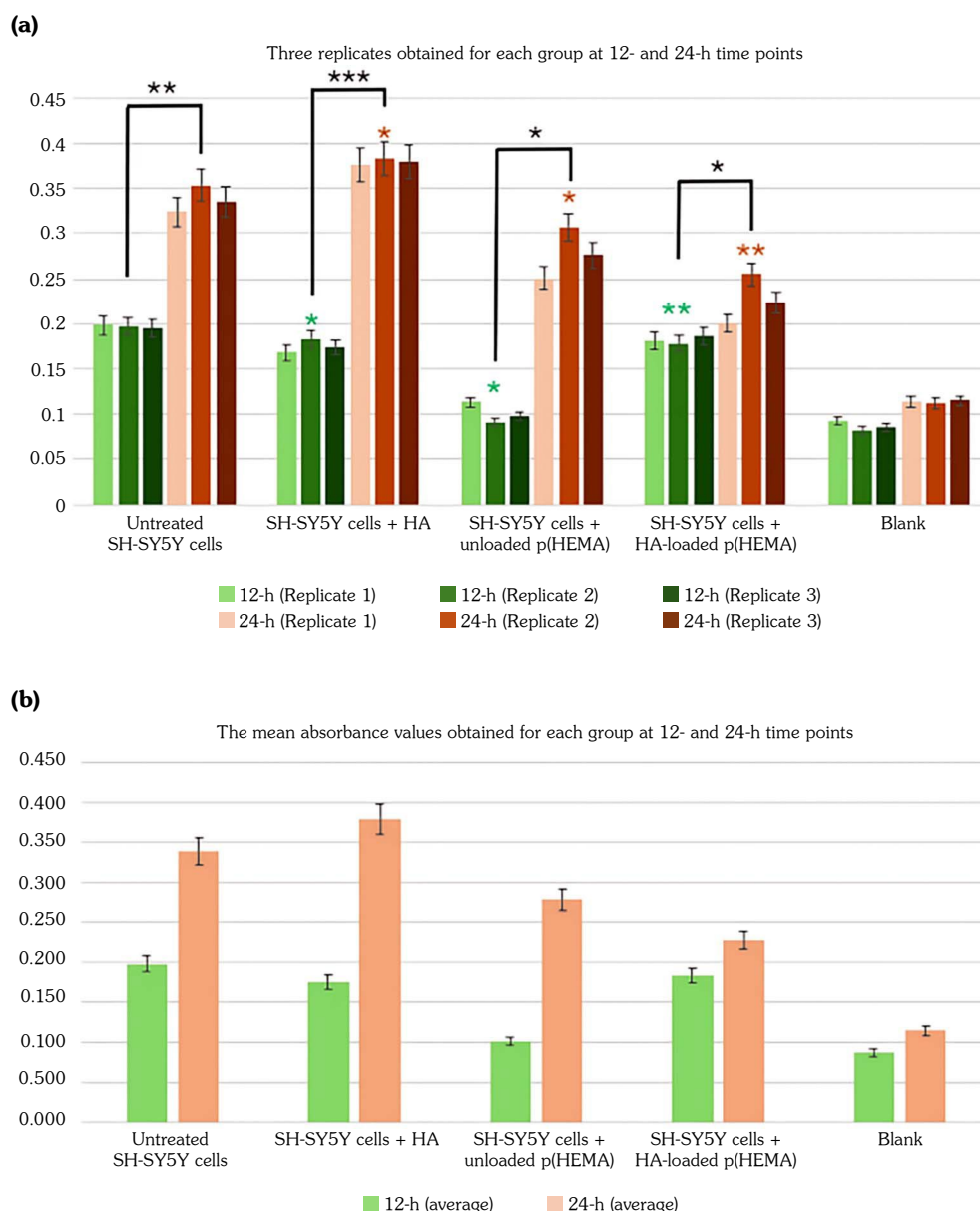


Figure 1. Viability of SH-SY5Y cells treated with HA-loaded p(HEMA) nanoparticles. **(a)** Individual data points from three technical replicates for each experimental group after 12- and 24-h incubation. These points represent the raw absorbance measurements used to calculate cell viability. **(b)** Bar graph showing the mean cell viability percentages \pm SD derived from the absorbance data. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test. Green and red asterisks denote significance levels versus the untreated control group at the same time point (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Black asterisks indicate significant differences between the 12- and 24-h time points within the same treatment group. The blank group was used to measure background absorbance.

HA: Hyaluronic acid; p(HEMA): Poly(2-hydroxyethyl methacrylate); SD: Standard deviation.

At the 24-h time point, the "SH-SY5Y Cells + free HA" group exhibited an increased mean absorbance of 0.3795, indicating that HA may promote cell proliferation over time. In contrast,

the "SH-SY5Y Cells + HA-loaded p(HEMA)" group showed a decreased absorbance value of 0.228, suggesting that the controlled-release HA-loaded p(HEMA) system may develop

cytotoxic effects over prolonged exposure and initiate targeted cell death (Figure 1b). The “SH-SY5Y Cells + unloaded p(HEMA)” group demonstrated an increased absorbance (0.279) compared to the 12-h measurement, which may indicate a reduction of the initially observed toxic effect over time or physiological adaptation of the cells to the environment (Table 2).

On the other hand, the low absorbance values observed in the blank group (0.087 and 0.113) were considered as background absorbance of the cell-free medium and were used as the reference values for calculating percentage viability (Table 2, Figure 1a, b).

DISCUSSION

Effects of the tested nanoparticle system on cell viability significantly vary depending on both the content and the duration of exposure. The results reveal that not only the presence of HA, but also the carrier system's presence and structure, can critically influence cellular behavior. Based on these data, the performance of the nanoparticle system is discussed below in the context of existing literature findings.

We evaluate the cytotoxic effects of HA-loaded p(HEMA) nanoparticles on the SH-SY5Y neuroblastoma cell line. According to cell viability data, the HA-loaded p(HEMA) system did not adversely affect cellular metabolism during short-term applications; however, a significant decrease in viability was observed after 24 h of incubation. These results suggest that the system's sustained-release profile may lead to time-dependent cytotoxicity by inhibiting cell proliferation. Consistent with the initial hypothesis of the study, the controlled release of HA via the carrier system was successfully demonstrated to reduce target cell viability.

Free HA increased metabolic activity, highlighting HA's supportive effect on cell viability. This finding aligns with previous studies reporting that HA, through its specific binding to CD44 receptors, can stimulate proliferative responses in certain cancer cells. For instance, Salari et al.^[7] and Luo et al.^[8] have demonstrated that

HA promotes cell proliferation via CD44 receptors on the cell surface. In this context, the data support the notion that free-form HA may create a cell-friendly microenvironment rather than exert a direct anticancer effect. However, in this study, when HA was presented together with the p(HEMA) matrix, the effect mechanism shifted over time, leading to the intended cytotoxic outcome. Cell viability initially decreased in the unloaded p(HEMA) group, and this decline continued until the 24-h mark. These findings suggest that p(HEMA) may initially inhibit cellular activity; however, cells seem to physiologically adapt to the material over time. One of the most notable findings of this study is that HA alone increased cell viability, whereas when integrated into a controlled-release system, it reduced viability over time. This result underscores the importance of evaluating both the carrier and the loaded agent in targeted drug delivery systems.

Furthermore, using p(HEMA) as a carrier allowed for the release of HA into the environment without directly changing its effect, thereby improving the system's biocompatibility. This study shows that HA-p(HEMA) based nanoparticle systems can produce increasing therapeutic effects on cancer cells over time.^[10,11]

Recent findings suggest that HA-loaded nanoparticles are internalized via endocytosis, after which intracellular degradation can lead to the activation of apoptotic pathways. Supporting this, demonstrated that hyaluronic acid HA-based nanoparticles could be visualized within intracellular compartments using Alcian Blue staining under transmission electron microscopy (TEM), confirming their localization to lysosomal vesicles and suggesting enzymatic degradation within the endolysosomal pathway.^[12] These visual data strengthen the notion that HA's intracellular fate, once encapsulated in a nanoparticle system, differs substantially from its free form and may underlie its time-dependent cytotoxic behavior observed *in vitro*.

Recent studies suggest that HA-loaded nanoparticles are internalized via CD44-mediated endocytosis, and once inside the

cell, they can induce reactive oxygen species-mediated apoptosis, significantly enhancing antitumor efficacy compared to free HA.^[13]

In comparative terms, similar cytotoxic trends have been observed with other HA-based delivery systems. For example, HA-coated chitosan nanoparticles have demonstrated effective targeting and heightened cytotoxicity in tumor models, emphasizing how the carrier's physico-chemical properties significantly affect therapeutic outcomes.^[14] These comparisons underline the importance of meticulously selecting the carrier matrix to modulate cellular responses effectively.

From a translational perspective, these results also indicate promising clinical applications for HA-p(HEMA) nanoparticle systems. Their ability to modulate cell viability over time could be used for targeted therapy, especially in intracranial tumors where overcoming the blood-brain barrier is a major challenge. Routes such as intrathecal or intranasal delivery could enable direct access to the CNS, bypassing systemic circulation.^[15] Additionally, the excellent biocompatibility and mechanical properties of p(HEMA) increase the system's potential for use in neurological drug delivery platforms.

Nevertheless, the fact that this study was conducted solely on the SH-SY5Y cell line and *in vitro* limits the generalizability of the findings. Comparative studies with different tumor cell lines, testing various dosages and release durations, as well as *in vivo* evaluations of the system, will better demonstrate the clinical application potential of the data.

In conclusion, adding HA to a nanoparticle-based delivery system not only changes its pharmacokinetic profile but can also significantly impact its biological effects. This study shows that p(HEMA)-HA systems may improve therapeutic responses over time in neuroblastoma cells, supporting their potential use for controlled-release, brain-targeted anticancer therapies.^[16]

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