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Research Article

Development of NGR-GelMA Hydrogels for PC3 Prostate Cancer Cells

Ziyşan Buse Yaralı Çevik[1*], Meryem Zeybekoğlu[2], Ozan Karaman[1,2]

[1] Biomedical Test, Calibration, Application and Research Center, Izmir Katip Çelebi University, Türkiye, ziysanbuse.yarali@ikcu.edu.tr
[2] Biomedical Engineering, Faculty of Engineering And Architecture, Izmir Katip Çelebi University, Türkiye, meryemzeybekoglu@gmail.com, ozan.karaman@ikcu.edu.tr
*Corresponding Author

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ABSTRACT

Prostate cancer is one of the most common cancer for men. Current therapies such as chemotherapy or radiotherapy non-spesifically affect cancerous cells. Current therapies need more targeted delivery approaches such as peptide. Asn-Gly-Arg (NGR) is a tool for cancer targeting therapy. To mimic more natural cancer microenvironment, peptide treatment approaches are examined in 3 Dimensional (D) hydrogels. GelMA is one of the hydrogels that permits to construct 3D microenvironment of PC3 prostate cancer cells. The goal of the study was to evaluate characteristic of GelMA to model prostate cancer environment and to determine the effects of NGR peptides for PC3 line. pH values of different concentrations NGR (1 µM, 10 µM and 100 µM)-GelMA were measured. To analyze biodegradation capacity of different concentrations NGR (1 µM, 10 µM and 100 µM)-GelMA, weigth measurements were performed. Live and Dead analysis was performed on days 1, 4, and 7. The findings revealed that GelMA hydrogels created a relatively stable and neutral pH, making them potentially valuable for drug delivery systems. Furthermore, the NGR-GelMA hydrogels incorporated exhibited the capacity to absorb liquids, resulting in an increase in weight. Notably, these hydrogels allowed for the observation of the dynamic 3D microenvironment of prostate cancer, which was influenced by the concentration of the targeted drug in the GelMA matrix. This suggests promising implications for developing targeted therapies for prostate cancer using GelMA-based drug delivery systems. As a conclusion, GelMA and NGR-GelMA hyrdogels may be useful platform for further studies to progress on prostate cancer treatment.

1. Introduction

Cancer has affected multicellular living organisms for more than 200 million years, and evidence of cancer among modern human progenitors dates back well over a million years [1]. Prostate cancer is the most frequent cancer among males in the Western world and the second largest cause of cancer mortality [2]. Prostate cancer is described as malignant tumoral formations caused by aberrant and uncontrolled growth of prostate gland cells, which are part of the male reproductive system [3]. Current chemotherapy and radiotherapy are used to fight cancers [4]. However, current therapeutic approaches have some disadvantages because of their high side effects and non-specific to target cells [5-7]. Thus, new methodologies have been developed to catch more specificity for cancerous region.

Peptides can specifically recognize the complex of antibody and cell surface [7, 8]. One of the first generation of tumor targetting peptides is the Asn-Gly-Arg (NGR) peptide [9]. The NGR peptide recognizes to aminopeptidase N (CD13),

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which is a receptor that is expressed on only tumor neovasculature [10]. To increase the anticancer action of several antitumor drugs as doxorubicin (dox), cisplatin, proapototic peptides, and tumor necrosis factor (TNF), NGR peptides have been utilized for their target delivery. Garde et. al. showed that NGR based dox showed reduction of tumor vasculature rather than only dox in PC3 prostat cancer cell line [11].

Anticancer targeting compounds including NGR motif can demonstrate higher affinity to tumor and promote higher efficacy. A study showed that only NGR can also affect and exhibit antitumor effect [12]. NGR peptides may also attach to CD13-v3+ tumor cells, including MDA-MB-435 breast cancer cells, and prevent them from migrating and proliferating in vitro condition [12].

Complex in vitro techniques are needed to mimic microenvironment of cancer tissues. 3 Dimensional (D) cell culture supports more natural biochemical signals and mimics tissue-specific architecture in terms of forced polarity, flattened cell shape, and subsequent cellular communication [13-16]. In recent years, the combination of hydrogel and peptide has been frequently used in 3D systems [17]. Hydrogel models may be successful in mimicking the tumour environment so hydrogel and peptide combination is a promising method for cancer studies [18]. Gelatin methacrylate (GelMA)-based hydrogels provide bioengineered, semi-synthetic 3-D platform for spheroid growth of ovarian cancer cells in vitro and in vivo [19].

Being unique features, such as biocompatibility, biodegradability, and adjustable mechanical properties, GelMA has a potential biomaterial for cancer therapy [20]. GelMA-based systems provide an excellent platform for the creation of cancer therapeutics and drug delivery systems. Since the effects of only NGR peptide and its different concentrations have not clarified, yet.

In this proof of the concept, the study aimed to examine usage of GelMA for PC3 prostate cancer modelling application and find the optimum concentration of different NGR peptide, one of the most important peptide for cell targeted approach, for PC3 prostate cancer cell line.

2. General Methods

All chemical and peptide products were purchased from Aapptec (Louisville, USA). GelMA was commercially taken from Zetamatrix, Türkiye. All cell culture materials were taken from Sigma Aldrich (St. Louis, Missouri, USA). Live and Dead was purchased from Dojindo (Munich, Germany).

2.1. NGR peptide synthesis

The Asn-Gly-Arg (NGR) peptide was synthesized on the Rink Amide NovaGel resin (0.62 mmol/g) [14, 16]. The resin was swollen with 3 mL dimethylformamide (DMF) solution for 30 min. Fmoc protected amino acid derivative (2 eq), HBTU (1.95 eq), and DIEA (3 eq) were dissolved in DMF. To remove the peptide sequence from the resin, the resin was then left in a trifluoroacetic acid (TFA)-based solution for 2 hours. The product was precipitated into cold ether and lyophilised.

2.2. Fabrication of GelMA and GelMA: NGR hydrogels

Lyophilised GelMA was dissolved in 45 °C deionised water to obtain 10% concentration prepolymer solution [21]. A photoinitiator (Irgacure-2959) at 0.5% concentration was added to the GelMA solution and the mixture was stirred at 70°C for 4 h to dissolve GelMA. Only GelMA hydrogel was used as the control group. 1 μM, 10 μM and 100 μM concentrations of NGR peptide were dissolved in GelMA.

After re moving air bubbles, the solutions were poured into a disposable 90 mm diameter culture. They were exposed to UV light for 2 min to cross-link only GelMA and NGR-GelMA. They were stored at 4 °C for further characterization as presented in Figure 1.

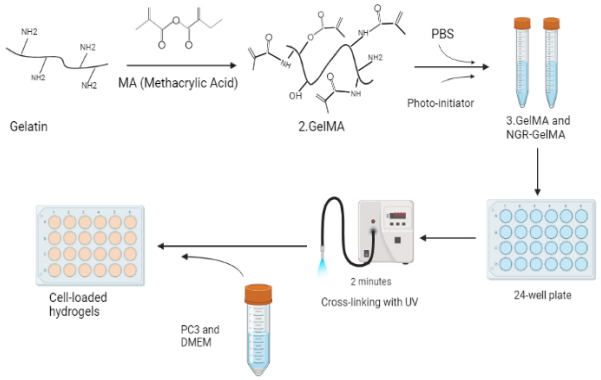

Figure 1. Basic experimental procedure for PC3 prostate cancer in GelMA and NGR- GelMA

2.3. pH and swelling analysis of GelMA

pH values in different concentrations NGR (1 µM, 10 µM and 100 µM)-GelMA were measured on days 1, 3, 5, 7, 9 and 12 to test acidity properties of these hydrogels. The dry weights of GelMA and NGR (1 µM, 10 µM and 100 µM)-GelMA hydrogels were measured on day 0 before placement in SBF. Then, the weights of GelMA and NGR (1 µM, 10 µM and 100 µM)-GelMA hydrogels were measured on days 1, 4 and 7.

2.4. Cell culture

PC3 cells were grown in DMEM medium including 10% FBS and 1% penicillin-streptomycin [14-16]. Cells were seeded in a 12-well plate at the density of 1 × 105 cells per well and maintained in an incubator with a humidified atmosphere containing 5% CO2 at 37°C. The culture medium was refreshed every two days. Cells were washed three times with DPBS and harvested by trypsinization.

2.5. Live and dead analysis of PC3 protate cancer cells

The Double Staining Kit was used to evaluate the viability analysis of PC3 prostate cancer. 1 mmol/L solution A-green (Calcein-AM/DMSO) and 1.5 mmol/L solution B-red (PI/pure water) were used get qualitative viability analysis of PC3 prostate cancer cells. The red colour represents dead cells and the green colour represents living cells. While the red colour represents dead cells, the green colour represents living cells.

2.6. Statistical analysis

All data are statistically evaluated using two-way ANOVA (SPSS 12.0, SPSS GmbH, Germany) and post hoc test. p values less than 0.05 were used to evaluate if there were significant differences between groups. (*p<0.05, **p<0.01, ***p<0.001).

3. Results And Discussion

GelMA is a biocompatible material which supports mechanical tunning [22, 23]. Bock et. al. utilized from GelMA, PEG and GelSH to create an organoid form for cancer cell lines [22]. They conjugated peptides to these hydrogels and concluded that functionalization of hydrogel with peptides may provide ex vivo organoid growth of cancer cells.

The pH values of NGR (1 µM, 10 µM and 100 µM)-GelMA hydrogels were measured. pH is important for gelation and cross-linking. Crosslinking of GelMA molecules is responsible for the formation of GelMA hydrogels. The pH value of the control group GelMA hydrogel showed values of 7.65± 0.2, 7.70± 0.2, 7.75± 0.2, 7.75± 0.2, 7.77±0.2, and 7.80± 0.2 on days 1, 3, 5, 7, 9 respectively as given in Figure 2.

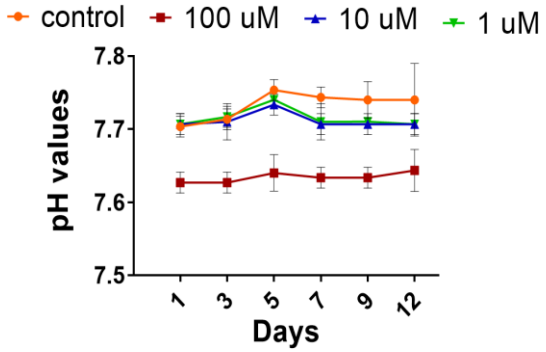

Figure 2. pH changing of only GelMA and NGR (1 µM, 10 µM and 100 µM)-GelMA hydrogels

pH of GelMA hydrogel increased depending on time, but remained constant at 7.80 after day 9. pH values of NGR (100 µM)-GelMA showed values of 7.63± 0.2, 7.63± 0.2, 7.63± 0.2, 7.63± 0.2, 7.63± 0.2, 7.65± 0.2 on days 1, 3, 5, 7, 9, and

12, respectively. pH values of NGR (100 µM)-GelMA remained constant over time, but started to increase on day 12. pH values of NGR (10 µM)-GelMA showed a higher pH value than the other experimental groups until day 7. On day 9, pH values of NGR (10 µM)-GelMA was observed as 7.77, while it was observed as 7.76 in NGR (10 µM)-GelMA.

On the 12 days, it was observed that the pH value of the control group and NGR (10 µM)-GelMA remained constant at 7.80. This showed that the pH values of only GelMA hydrogel and NGR (10 µM)-GelMA continued to increase until the 9th day. pH values of NGR (1 µM)-GelMA were 7.70± 0.2, 7.75± 0.2, 7.76± 0.2, 7.77± 0.2, 7.76± 0.2, 7.76± 0.2, 7.80± 0.2 on days 1, 3, 5, 7, 9, and 12, respectively. This showed that the pH value of NGR (10 µM)-GelMA increased over time but remained at a constant value (7.80) after day 9. In this study, pH measurements of GelMA hydrogels and NGR (1 µM, 10 µM and 100 µM)-GelMA hydrogels fluctuated over time, the pH value stabilised at 7.70 on the 12nd day. The pH value of NGR (1 µM)-GelMA hydrogel was 7.63 on day 1, which was lower than the other experimental groups. The pH value gradually increased over time and reached 7.70 on day 12. GelMA in the control group and different concentration NGR (1 µM, 10 µM and 100 µM)-GelMA hydrogels created a slightly basic environment.

GelMA and NGR-GelMA hydrogels have been investigated for drug delivery applications. The pH sensitivity of the hydrogel can be utilised to design pH-sensitive drug delivery systems [24, 25]. For example, the hydrogel can undergo controlled degradation for tumour tissue or cause the release of charged therapeutic agents. pH values of GelMA clearly affects drug release kinetics, possibly as a result of the requirement for extra diffusion across the GelMA layer, which lengthens the duration of the release [25].

Gelation kinetics and crosslinking efficiency can be affected by the pH of the GelMA precursor solution. Proper hydrogel formation and mechanical stability can be achieved by optimising the pH during gelation. Moghdateri concluded that high acidity promotes drug releasing. In addition, Pan et al. also showed that the pH values of GelMA changed between 6.4 and 8.4 [24]. Literature also concluded that pH can affect the stability and activity of proteins and enzymes in hydrogels [24, 26].

However, healthy cell viability and behavior can also be affected by the pH of the hydrogel [24]. It is critical to maintain a suitable pH range to promote cell adhesion, proliferation and differentiation within the hydrogel matrix [24, 25].

pH values should be compatible with the physiological environment and promote the integration of the hydrogel into the host system. The basic pH of environment can suppress the growth and proliferation of cancer cells while promoting the growth of healthy cells [27]. Raghunand et al. concluded that pH and drug resistance associated each other in tumours and mentioned that low extracellular pH in tumours contributes to drug resistance [28]. Gu et al. showed that pH values of liposomes hardly ever change when NGR peptide bond the liposomes [29].

As peptides are able to change biodegradability of modified materials, dry weights of NGR-GelMA were measured on days 0, 1, 4 and 7 as shown in Figure 3.

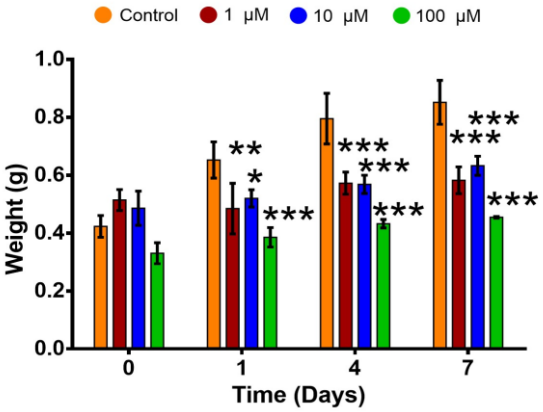

Figure 3. Weight measurements of only GelMA and NGR-GelMA hydrogels

Briefly, the initial weights of the hydrogel scaffolds were measured. The weights of the control group GelMA hydrogel were measured as 0.423± 0.02 g, 0.653± 0.04 g, 0.796± 0.09 g and 0.852± 0.00 g on days 0, 1, 4 and 7, respectively. This shows that the weight of GelMA hydrogel increases with time depending

on liquid absorbing capacity. The weights of NGR (100 μM)-GelMA were measured as 0.331± 0.2 g, 0.386± 0.2 g, 0.433± 0.2 g and 0.455± 0.2 g on days 0, 1, 4 and 7, respectively. This showed that NGR (100 μM)-GelMA absorbed into the liquid and increased its weight. The control group was found that it had the lowest weight compared to GelMA and other experimental groups.

The weight of NGR (100 μM)-GelMA was initially less than the other experimental groups so it may be the reason. It was also thought that the absorption potential of NGR (100 μM)-GelMA was less than the other experimental groups. The weights of NGR (10 μM)-GelMA were measured as 0.486± 0.2 g, 0.520± 0.2 g, 0.569± 0.2 g, 0.633± 0.2 g on days 0, 1, 4 and 7, respectively. This indicated that the weight of NGR (10 μM)-GelMA increased with time. The results showed that the NGR (10 μM)-GelMA was absorbed by the liquid and increased its weight. The weights of NGR (1 μM)-GelMA were measured as 0.514± 0.2 g, 0.485± 0.2 g, 0.573± 0.2 g, 0.583± 0.2 g on days 0, 1, 4 and 7, respectively. The weight of NGR (1 μM)-GelMA increased with time. The results showed that NGR (1 μM)-GelMA increased its weight by absorbing into the liquid. The results show the variation of the weights of GelMA hydrogels and NGR (1 μM, 10 μM and 100 μM)-GelMA over a period of time (on days 0, 1, 4 and 7).

The weights increased continuously with time, indicating that the hydrogels absorbed liquid from the surrounding medium. The reason of these weight changes is the hydrophilic nature of GelMA hydrogel [30]. When GelMA hydrogel was incubated in simulated body fluid for this study, it started to absorb the fluid through a process called imbibition. The porous structure of the hydrogel allows it to absorb and retain water, the weight increased. This absorption occurs due to the presence of hydrophilic functional groups in the GelMA structure that have affinity for water molecules. NGR (1 μM, 10 μM and 100 μM)-GelMA also show their ability to absorb liquid by showing weight increase over time. However, it was observed that the NGR (100 μM)-GelMA had the lowest weight compared to the control group GelMA and other experimental groups. This may be due to the relatively low initial dry weight of the NGR (100 μM)-GelMA, suggesting that the GelMA material had a lower mass before the absorption process begins. Overall, the weight changes in GelMA hydrogels and NGR (1 μM, 10 μM and 100 μM)-GelMA were primarily due to the hydrophilic nature of GelMA and its ability to absorb liquid by sorption. Specific concentrations of NGR can affect the initial weight and absorption potential to some extent as observed in the experiment.

On days 1, 4 and 7, PC3 prostate cancer cell line was encapsulated into NGR (1 μM, 10 μM and 100 μM)-GelMA. Live/Dead staining analysis was performed to determine the viability. As a result of the analysis the images obtained with the microscope are shown in Figure 4.

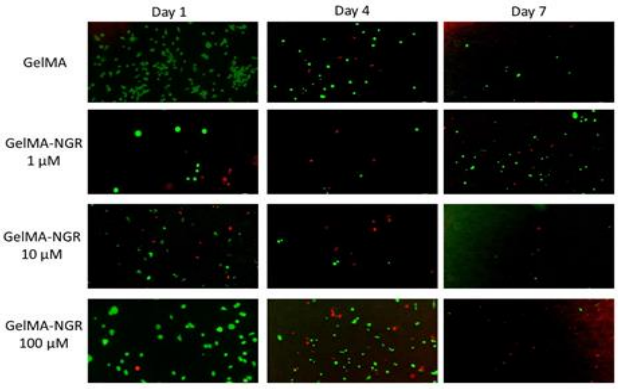

Figure 4. Images of the live and dead analysis of PC3 cell line in the control group GelMA and NGR (1 μM, 10 μM and 100 μM)-GelMA on days 1, 4 and 7

In Figure 4, green colour represents living cells and red colour represents dead cells. Only GelMA was used as a control group. The control group observed higher green colour intensity and less red colour intensity compared to NGR-GelMA hydrogel on days 1 and 4. It indicated that the number of living cells was higher as the green colour was higher in NGR-GelMA. However, the green colour intensity in only GelMA hydrogel was relatively less than NGR (1 μM)-GelMA hydrogels at day 7.

At day 7, NGR (1 μM)-GelMA hydrogels showed that the the number of viable cells was less than the control group. In NGR (100 μM)-GelMA, the green colour intensity at day 1 and day 4 was relatively higher than the red colour

intensity. However, on day 7, the red colour intensity was higher than the green colour intensity. This indicated that NGR (100 μM)-GelMA relatively proliferated on day 1, but the number of dead cells increased after day 4. These results show that NGR (100 μM)-GelMA hydrogel did not provide a favorable environment for the survival of the cells, it contained and increased the number of dead cells.

Changes in PC3 cell viability over time were observed in the results of live/dead staining analysis of GelMA and NGR (1 μM, 10 μM and 100 μM)-GelMA hydrogels. The only GelMA hydrogel provided a favorable environment for cells. Cell surviving and growing, which is reflected in living cells, observed on days 1 and 4. However, the green colour intensity in GelMA hydrogels decreased on day 7. This may be due to limitations in nutrient diffusion or accumulation of metabolic waste within the hydrogel over time. Similarly, Shih et al. in 2019 investigated the effect of oxygen diffusion on cell viability in the hydrogel [31].

The results showed that the transportation ability of oxygen and nutrients in the hydrogel to cells and removing metabolic wastes are limited so cell viability decreased over time due to the limitations of oxygen diffusion in the hydrogel [32]. Miri et. al. used MCF-7 line to model the breast cancer in GelMA [32]. They proved that nutrients are able to diffuse into breast cancer cell line. The conclusion was coincided with the behavior of control group GelMA [33]. Fussion proteins within different concentration of NGR sequence demonstrated cytotoxic effects on cancer cell lines and reduced cell survival [34]. Since NGR (1 μM)-GelMA included the highest red cells and lowest green cells on day 7, the concentration may have been effective in promoting cell apoptosis and suppressing cell viability in the hydrogel. The dynamic nature of cell behavior and the microenvironment within hydrogels may explain the observed differences in cell viability between time points. The decrease of cell viability in NGR (1 μM) -GelMA hydrogels may indicate prolonged exposure to the hydrogel environment at day 7. GelMA presented high producibility and controllability for PC3 prostate cancer cells. It is possible to observe changing 3D microenvironment of PC3 prostate cancer depending on targeting drug concentration in GelMA.

The results appeared the effects of different concentration NGR (1 μM, 10 μM and 100 μM)-GelMA in terms of PC3 prostate cancer viability. These findings suggested that it is important to optimize NGR (1 μM, 10 μM and 100 μM)-GelMA hydrogels to achieve the desired results in PC3 prostate cancer cell studies. Further research is needed to understand the underlying mechanisms and explore the potential of NGR-GelMA hydrogels as a therapeutic approach for prostate cancer treatment.

4. Conclusion

GelMA hydrogels provide a neutral pH environment that remains relatively stable, which may be effective for drug releasing systems. NGR-GelMA hydrogels were able to absorb liquid and their weight increased. GelMA hydrogels showed promise as a suitable matrix for PC3 prostate cancer cells. To calibrate model constants, experimental factors like cell line, testing duration, and peptide modification should be changed to match the intended volume of GelMA. In case of usage of NGR (100 μM) may contribute to PC3 prostate cancer death.

Further research and optimisation is required to fully understand the underlying mechanisms and potential applications of GelMA and NGR-GelMA hydrogels in PC3 prostate cancer therapy.

Article Information Form

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Authors' Contribution
Z.B.Y.Ç. designed the study. Z.B.Y.Ç. and M.Z. performed all experiments. Z.B.Y.Ç. and O.K.

concluded and funded the experiments. All authors wroted, proofread, revised and approved the manuscript.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics

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