Chitosan-Glycerol Hydrogel

Robert Kobrin

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Abstract:

The purpose of the research conducted through BME 295 was to create an injectable hydrogel for the intratumoral delivery of immunotherapeutics. A novel physically crosslinked chitosan-glycerol injectable gel was developed to improve therapeutic retention for intratumoral delivery. Different relative glycerol to chitosan volumes, ranging from 5 to 95% glycerol, were tested to determine the optimal gelation conditions for a variety of chitosan types. Phosphate-buffered saline was incorporated into the solution, which was then raised to a pH of 7.8 with sodium hydroxide. After centrifugation at 10,000 rpm for 5 minutes, chitosan-glycerol gel was formed. Glycerol most significantly impacted gelation conditions independent of chitosan concentration, with gelation occurring between 70-85% relative volume glycerol. Chitosan viscosity also had a significant impact. The gel was injected through a 25-gauge needle into gelatin-based tumor phantoms, and it displayed increased load retention time compared to various saline and chitosan-based solutions. Due to this increased retention time, chitosan-glycerol gel offers an effective platform for intratumoral delivery of immunotherapeutics.

Introduction:

Localized cancer therapeutic delivery provides several advantages over systemic delivery, including increased retention and reduction of systemic side effects [1]. In particular, intratumoral injections are an advantageous delivery method for cancer treatment. However, the dense extracellular matrix and higher pressure tumor environment severely limit injection retention, as less viscous solutions easily return via the needle track [2]

Figure 1: Dyed saline solution demonstrating poor injection retention 5 minutes after delivery

. Gel delivery systems address this limitation due to their increased viscosity and hence improve retention at the tumor site, allowing for the long-term, controlled release of therapeutics. Chitosan, a polysaccharide derived from shells and scales of crustaceans and fish, has been used

in gel creation due to its wide availability and its biocompatible nature [3]. Glycerol, a thickening/smoothing agent often used in food and cosmetic applications, has been used in hydrogels due to its biocompatibility and its ability to improve viscosity and mechanical properties [4].

Methods (Gel Creation):

The gel creation process of the chitosan-glycerol gel centered around the combination of chitosan solution and glycerol. First, chitosan was dissolved in 0.1M HCl due to its solubility in dilute acids. This solution was then combined with a large relative volume of glycerol before being buffered via the addition of 1X PBS saline solution. The resulting solution was neutralized to physiological pH with 1M NaOH, prompting initial gelation. Finally, the solution was centrifuged for five minutes at 4200 rpm, leading to further gelation upon the drainage of the resultant remaining solution.

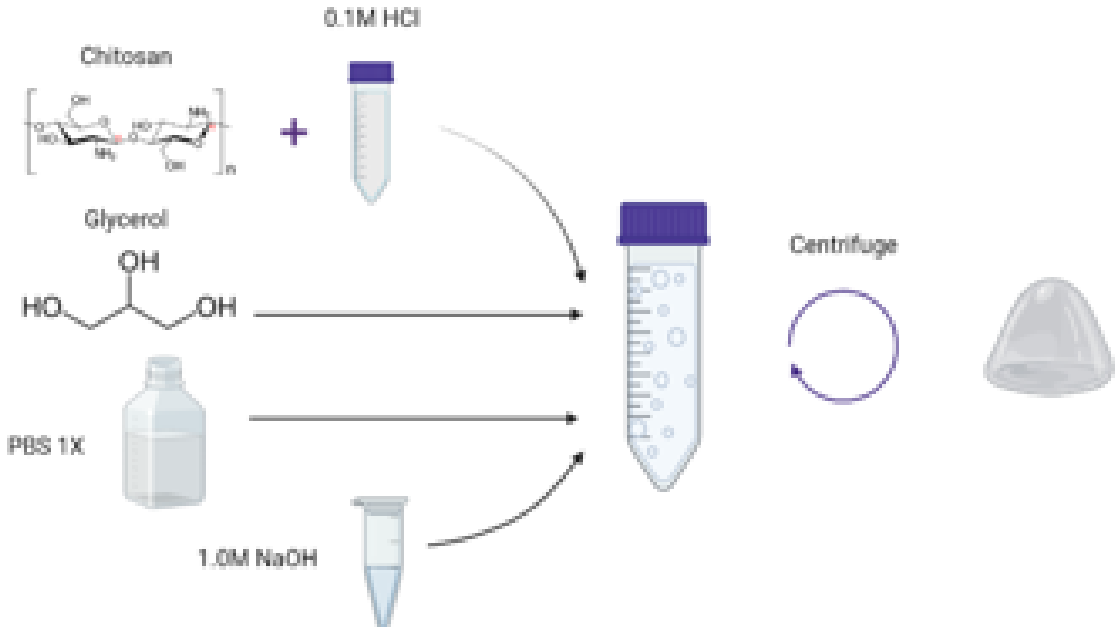

Figure 2: Graphic demonstrating the creation process of the Chitosan-Glyerol Gel

Upon completion of the centrifugation, the created hydrogel was saturated due to its presence in the remaining solution. Dehydration of the created gel led to an unsaturated hydrogel displaying increased viscosity and decreased ease of injectability.

Methods (Parameterization):

The parameterization of the chitosan-glycerol gel centered around the testing of three variables: chitosan-glycerol ratio, chitosan volume, and chitosan type. Testing involved the slight variation of single aspects of the chitosan-glycerol gel creation process in order to isolate variables and draw conclusions based on the resulting gels.

The first gelation parameter testing involved the chitosan-glycerol ratio. To do this, 70/100 chitosan solution was created in 0.1M HCl in three different concentrations (10 mg/mL, 15 mg/mL, and 20 mg/mL). The gel creation process detailed in the methods section was then followed, with the only variation occurring in the ratio of chitosan solution to glycerol used. Ratios of 95:5 glycerol to chitosan solution to 5:95 glycerol to chitosan solution were tested with differences of 5 percent between the benchmarks. This involved the creation of 57 trials (19 per chitosan solution concentration). Upon the conclusion of the gel creation process, the created solutions were examined for evidence of precipitate formation or hydrogel formation. Precipitates were characterized as viscous substances unable to hold their form through the application of motion, while gels were characterized as viscous substances able to hold their form through the application of motion.

The section gelation parameter tested was chitosan type. To do this, chitosan solutions were created at a constant concentration of 15 mg/mL in 0.1M HCl using nine varying types of chitosan. The chitosans involved had degree of acetylation numbers of 70,80, and 95, with each type having three types representing viscosity numbers of 5,100, and 2000. A similar procedure to the previous parameter testing was employed, with varying chitosan:glycerol ratios employed in order to determine the gelation parameters of different types of chitosan at constant chitosan concentrations. Upon the conclusion of the gel creation process, the created solutions were examined for evidence of precipitate formation or hydrogel formation. Precipitates were characterized as viscous substances unable to hold their form through the application of motion, while gels were characterized as viscous substances able to hold their form through the application of motion.

Methods (Retention Testing):

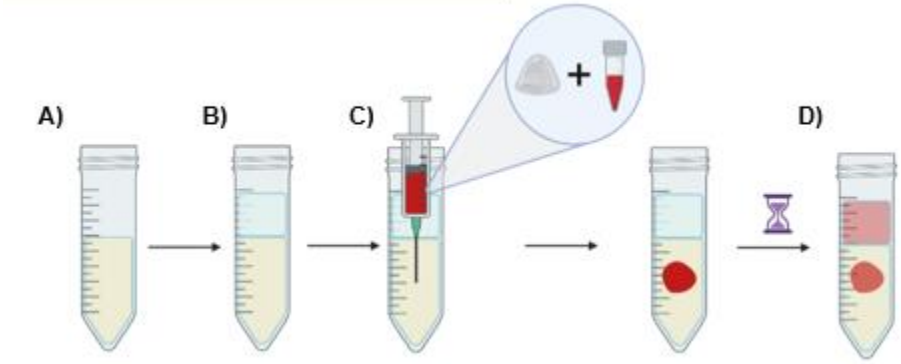

Figure 3: Visual depiction of the conducted retention testing experiment. ***A****) Agar-based mechanical tumor phantom.* ***B****) Loaded 1X PBS to provide a base for red dye absorbance testing* ***C****) Injection of solutions containing constant volumes of red dye at a constant depth into tumor phantoms* ***D****) Red dye-loaded injections leak out of injection site, increasing red dye absorbance in loaded 1X PBS*

The goal of the project was to create an injectable gel that displayed increased retention of immunotherapeutics compared to the lab standard injection of 1.5% w/v chitosan solution. In order to test the improved retention of the created chitosan-glycerol gel, a retention experiment was run to compare the gel to various injection standards. Mechanical tumor phantoms were created using agar in deionized water to simulate the pressure environment of a tumor. Three rounds of injections were prepared: 1X PBS saline solution, lab standard 1.5% w/v chitosan solution in 0.1M HCl, and the chitosan glycerol gel. These injections were saturated with a constant volume of red food dye. An absorbance standard was then taken of the red food dye. Tumor phantoms were loaded with 10 mL of 1X PBS solution above their surface in a 50mL centrifuge tube. The prepared injections were then loaded into the tumor phantoms. Red dye absorbance was measured from the 1X PBS solution loaded above the tumor phantoms over the span of 24 hours in order to determine the red dye leaving the injection site via the needle track.

Methods (Cell Viability Studies):

In order to be viable as an immunotherapeutic delivery system, the created gel must display biocompatability. To prove biocompatibility, a cell viability study was done using cell counting via trypan blue dying. 3T3 embryonic cells were prepared and cultured. 100,000 cells

were then allocated to each well of a 24-well plate. A positive control of 1M NaOH was used to display cell death, while a negative control of an untampered experiment group was used to further validate the experiment. Experimental groups comprising of 6 well plates each were then loaded with varying volumes of the chitosan-glycerol gel. Cell counting was performed at set time periods over 48 hours using trypan blue cell dying. The cell counts were then compared to the pre-experiment cell count in each well in order to determine viability.

Results (Parameterization):

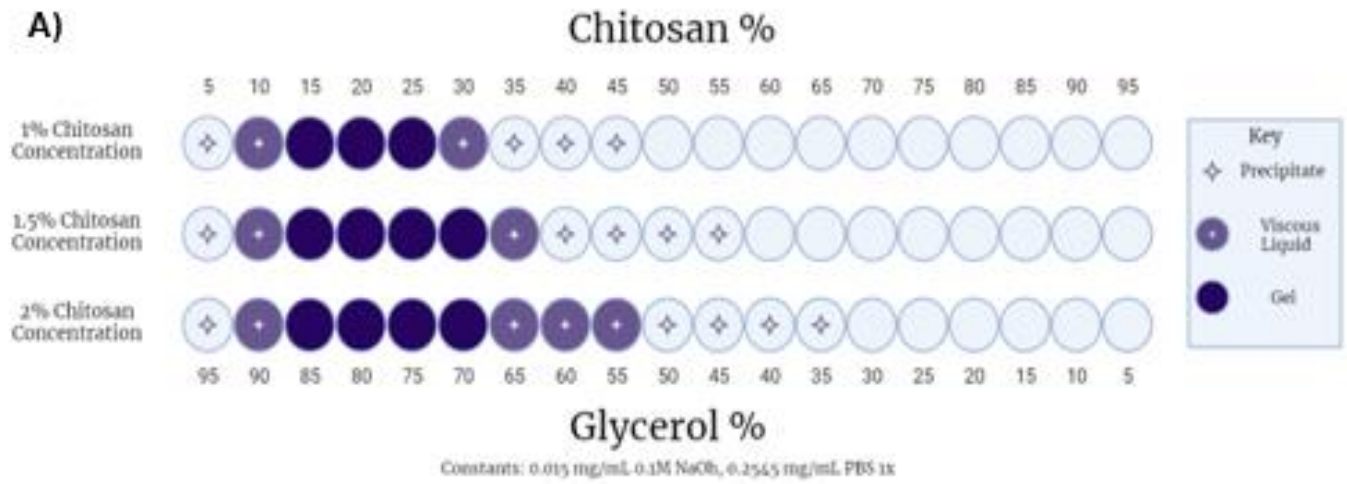

Figure 4: Chitosan-Glycerol parameterization figure demonstrating the isolation of the chitosan-glycerol concentration ratio variable

The first parameterization experiment focused on the role of the ratio of chitosan solution to glycerol in the gelation of the created gel. When using 70/100 chitosan of varying concentrations, a gelation zone of 15-25% chitosan to 85-75% glycerol was found to be optimal for gelation. The varying concentration of the chitosan solely impacted the range of precipitates created.

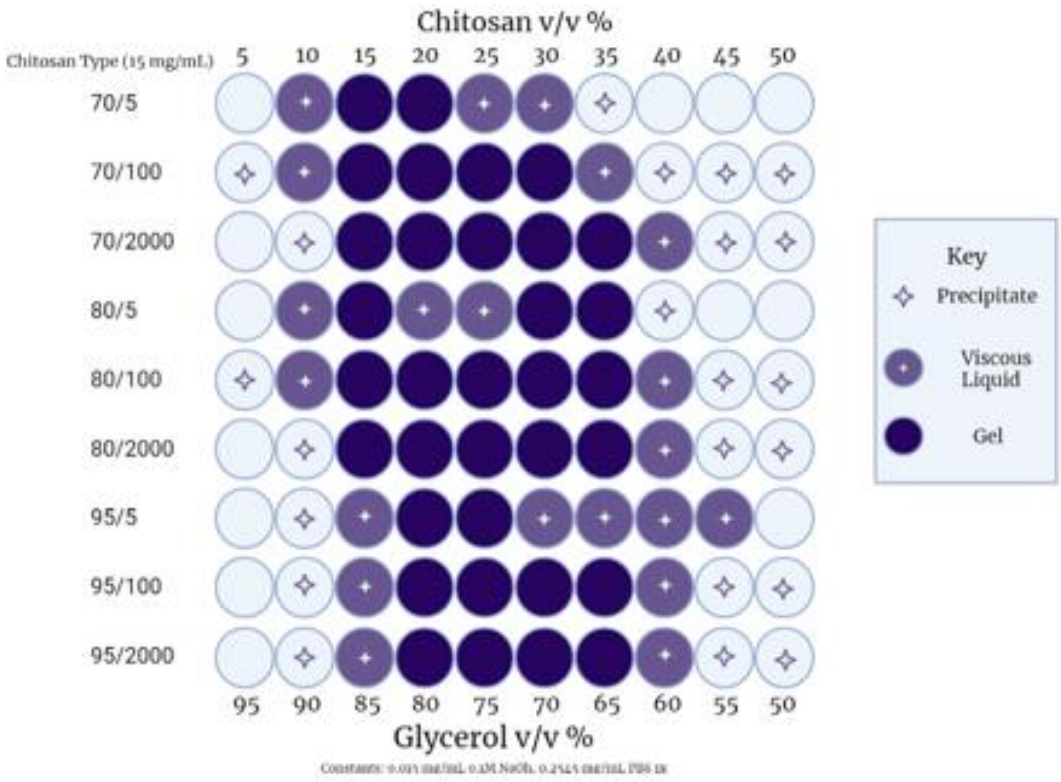

Figure 5: Chitosan-Glycerol parameterization figure demonstrating the isolation of the chitosan type variable

The second parameterization experiment served to isolate the variable of chitosan type. The data presented by the experiment, shown above, depicts a minor effect of the degree of acetylation on the gelation parameters of the chitosan-glycerol hydrogel and a major effect of the viscosity number. The degree of acetylation of the chitosan type used demonstrated only a minor effect, as the main gelation trends remained the same between chitosan types of equal viscosity numbers and chitosan-glycerol ratios over different degrees of acetylation. However, the viscosity number of the chitosan demonstrated a major effect on the gelation parameters. Chitosan of low viscosity number (5) demonstrated reduced gelation, with slimmer gelation parameters and a larger range of precipitates created. Chitosan of high viscosity number (2000) demonstrated an inability to create a homogenous solution, leading to problematic gels with varying qualities. This points to the existence of a zone of chitosan viscosity that promotes optimal gelation.

Results (Retention):

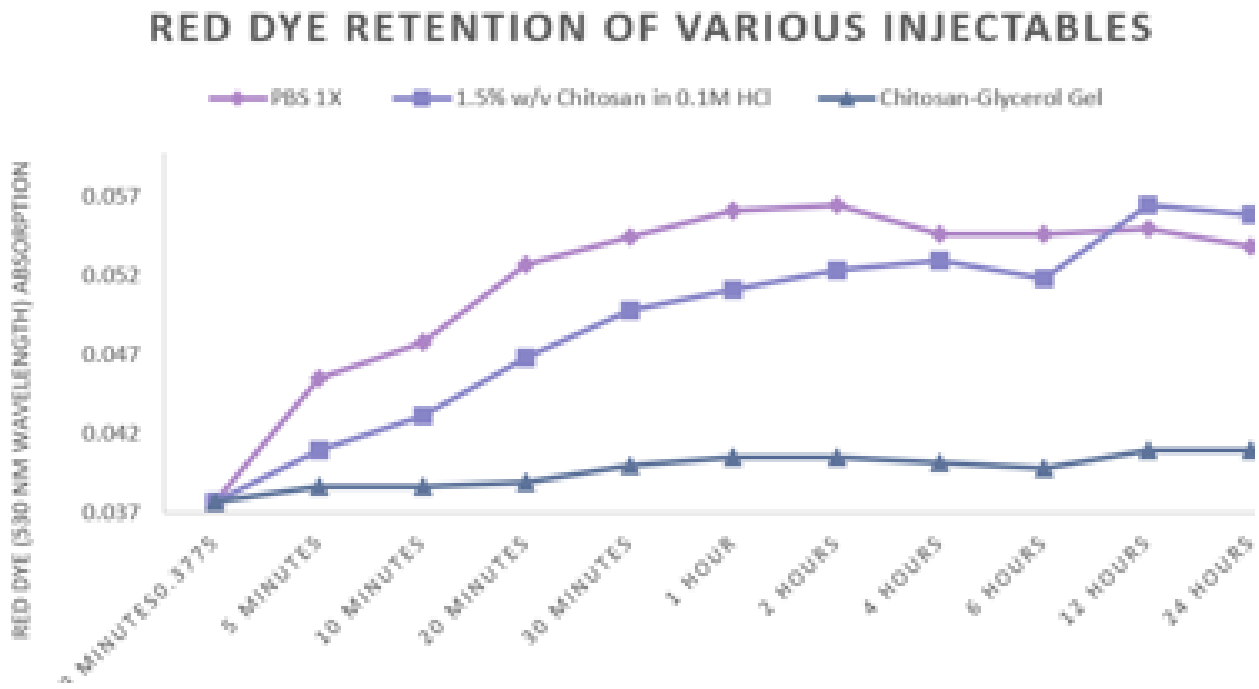

Figure 6: Red dye retention of three series of injections over a 24 hour time period

The injection retention testing demonstrated the vastly increased retention of the chitosan-glycerol hydrogel over the lab standard 1.5% w/v chitosan solution and the saline solution. Over the course of 24 hours, the chitosan-glycerol hydrogel demonstrated high retention, with only a slim portion of its loaded red dye escaping through the needle track into the 1X PBS saline solution above. The lab standard chitosan solution and saline solution injections displayed poor injection retention in comparison, with the majority of the loaded red dye present in the saline solution above the injection site after 24 hours.

Discussion:

Over the course of the BME 295 timeframe, an injectable hydrogel was created from chitosan and glycerol that demonstrates many desirable qualities. The chitosan-glycerol gel is injectable through a 25-gauge needle, demonstrates biocompatibility through initial testing, and shows vastly improved load retention over lab standard chitosan solution injections. While the gel requires further testing in order to understand the role hydration plays in its mechanical properties as well as to confirm its biocompatibility, its promising aspects warrant the further

research. Future project directions include finding a specific immunotherapeutic, most likely a protein or a cell, that can be loaded into the gel to create a working vaccine, as well as improving understanding of the injection of the gel through animal trials that better mimic human *in vivo* activity.

Sources:

[1] Eccleston DS et al. *Rationale for local drug delivery*. Semin Interv Cardiol. 1996 Mar;1(1):8-16. PMID: 9552480.

[2] H.-gi Kim, A. R. Yu, J. J. Lee, Y.-J. Lee, S. M. Lim, and J. S. Kim, "Measurement of tumor pressure and strategies of imaging tumor pressure for Radioimmunotherapy," *Nuclear Medicine and Molecular Imaging*, vol. 53, no. 4, pp. 235–241, 2019.

[3] Nilsen-Nygaard J et al. Chitosan: Gels and Interfacial Properties. *Polymers*. 2015; 7(3):552-579. https://doi.org/10.3390/polym7030552

[4] Y. Xia, Y. Wu, T. Yu, S. Xue, M. Guo, J. Li, and Z. Li, "Multifunctional glycerol–water hydrogel for biomimetic human skin with resistance memory function," *ACS Applied Materials & Interfaces*, vol. 11, no. 23, pp. 21117–21125, 2019.