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pH-DEPENDENT RNA ISOLATION FROM CELLS ENCAPSULATED IN CHITOSAN-BASED BIOMATERIALS

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Abstract

Chitosan has emerged as a useful biomaterial employed in tissue engineering and drug delivery applications due to its tunable and interesting properties. However, chitosan is protonated at biological pH and thus carries positive charges, which renders chitosan incompatible with conventional methods of RNA extraction. RNA extraction is an important step in investigating cell responses and behavior through studying their gene expression transcriptional profile. While some researchers have tried different techniques to improve the yield and purity of RNA extracted from cells encapsulated in chitosan-based biomaterials, no single study has investigated the effects of manipulating pH of the homogenate during RNA extraction on the yield and quality of total RNA. This study confirms the release and binding of RNA from chitosan to be pH dependent while analyzing the impact of pH changes during the tissue disruption and homogenization step of extraction on the resulting yield and quality of isolated RNA. This concept was applied to three commonly used methods of RNA extraction, using adult neural stem cells (aNSPCs) encapsulated within methacrylamide chitosan (MAC) as model chitosan-based bioscaffold. High pH conditions resulted in high yields with good quality using both TRIzol and CTAB. pH of the homogenate did not affect RNeasy spin columns, which works best in neutral conditions with good quality, however, the overall yield was low. Results in total show that pH affects RNA interaction with a chitosan-based bioscaffold, and thus alters the concentration, purity, and integrity of isolated RNA, dependent on the method used.

Graphical Abstract

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Keywords

RNA extraction; Chitosan; Methacrylamide chitosan; pH; CTAB; TRIzol

1. Introduction

Cell-polymer scaffolds were first introduced as a cell transplantation strategy by Vacanti et al. [1] and remained a key strategy in regenerative medicine approaches. Hydrogels represent an important class of bioscaffolds due to their appealing properties, such as the ability to protect encapsulated cells against immune mechanisms [2], while allowing free diffusion of oxygen and other nutrients. The ability to allow for modification of physical and chemical properties has allowed researchers to use hydrogels in combinations tailored for a wide range of tissues and cells [3–5]. Structurally, hydrogels are chemically or physically cross-linked networks of polymers with high water content [6]. Hydrogels can be classified based on the material used for their formation into natural, synthetic, and semi-synthetic. Polypeptides and polysaccharides are a subset of natural polymers that are also used to form natural hydrogels [6].

Chitosan is a naturally abundant polysaccharide often used as a biomaterial. Chitosan-based hydrogels are widely used in tissue engineering (TE) and drug delivery because of several advantages. The primary amine groups in chitosan's structure allow for multiple modifications and functionalization approaches. Specifically, researchers can easily engineer the physical properties of chitosan and functionalize it with various growth factors and cues to mimic the extracellular matrix (ECM) and other tissue microenvironments [7]. Importantly, low molecular weight chitosan is known to inhibit RNase activity; thus it helps protect RNA against degradation [8]. However, the poor solubility of chitosan in aqueous media limits its applications. In order to be solubilized natively, chitosan requires acidic solvent [4,9], which leads to protonation of its amine group making it positively charged. Previously in our group, modified chitosan has been used to mimic central nervous system (CNS) ECM to provide an essential environment for aNSPCs to differentiate into various types of neural cells [2,10].

Cells are the second most crucial component of any TE approach. It is vital to understand the biological response of cells encapsulated in hydrogels to be able to evaluate the effects of incorporated factors on cell behavior. Cell behavior is often reflected in gene expression, which can be studied using various techniques, such as quantitative reverse transcription PCR (RT-qPCR) and microarray analysis. Regardless of the technique used, high quality and purity RNA with good yield is the first and most important step toward PCR-based assays to explain cell behavior. RNA is a single-strand nucleic acid that is ionic in physiological pH due to the phosphate group in its backbone [11]. The ionic nature of RNA imposes a challenge with chitosan-based biomaterials since the cationic chitosan causes electrostatic interactions that make it difficult to isolate sufficient amounts of high-quality RNA from cells encapsulated within these materials. Conventional methods of RNA isolation are not capable of overcoming these electrostatic interactions, which result in precipitation of RNA or contamination of RNA with chitosan fragments.

There is a need to establish a simple method for RNA isolation from cells encapsulated in chitosan-based biomaterials because these biomaterials are extensively used in regenerative medicine applications. Current RNA isolation methods include phase separation or solid-state binding techniques [12], with the homogenization of tissue as the first step in both techniques. However, the reagents used for lysis and homogenization differ from one technique to another. After homogenization, the first set of techniques employ a phase separation principle to separate RNA, DNA, and protein into the aqueous, organic, and interphase, respectively [13]. In the phase separation technique, RNA is collected by precipitation of the aqueous phase, typically using isopropanol. On the other hand, the solid-state binding (*e.g.*, spin-column) technique involves the use of silica membrane that binds to RNA in the presence of high ionic strength solutions. After washing other contaminants, a low ionic strength solution is used to elute RNA from silica membranes [12]. Silica membranes are negatively charged and can bind the negatively charged RNA readily via a cationic bridge of sodium ions added to buffers during processing [14]. However, if positively charged chitosan is present, it can be competitive in this binding process, leading to lower RNA yields. Moreover, nucleic acid binding and release from chitosan have been previously reported to be pH-dependent [15], and researchers have demonstrated RNA purification in a microfluidic device relying on the pH-dependent release of RNA from chitosan [16]. The present study was designed to utilize a similar approach in terms of manipulating pH, targeting the pH of the tissue homogenization solutions of the three common RNA extraction methods. We hypothesized that changing the pH conditions of the homogenization solution would improve the yield, purity and integrity of extracted RNA. The mechanism proposed allows for reduced ionic interactions to allow the release of RNA from chitosan during the homogenization stage, which allows for precipitation of chitosan without RNA entrapment to increase the yield of RNA extraction. To evaluate this approach, the effects of homogenization pH were studied by assessing RNA yield, purity and integrity. Also, since our ultimate goal was to use the RNA isolated through this approach in downstream applications, the RNA was tested using RT-qPCR as a functional readout.

2. Experimental

2.1 Methacrylamide Chitosan (MAC) Preparation

N-Methacrylation of chitosan is the method that has been adopted in our lab after it has been used and characterized by Li et al. [10] based on a previous method by Yu et al. [17] and it is summarized in Fig. 1. Briefly, chitosan (Protosan UP B80/20, NovaMatrix, Drammen, Norway) was dissolved in 2% (v/v) acetic acid at a final concentration of 3 wt%. Methacrylic anhydride (Sigma-Aldrich, St. Louis, MO, USA) was then added at 1 : 0.4 molar ratio (chitosan : methacrylic anhydride) and allowed to mix magnetically for 3+ h at RT. After complete dissolution, the mixture was dialyzed (12–14 kDa dialysis tubing, Spectrum Labs, Waltham, MA, USA) against distilled water for 72 hours. Following dialysis, samples were freeze-dried and stored at –20 °C until use.

2.2 aNSPC Harvest and Culture

All procedures that involved animals were approved by the University of Akron institutional animal care and use committee (IACUC). Our lab has adopted a well-established protocol to harvest neural stem cells from SVZ of the lateral ventricles of adult rat brain. This protocol uses selective media to isolate aNSPCs from primary neural cells and grow them in clusters, called neurospheres [18]. First, adult female Fischer 344 rats (6–8 weeks old, Envigo, Haslett, MI, USA) were euthanized using CO₂ followed by decapitation. Under sterile conditions, the brain is entirely removed using spatula and forceps. The two hemispheres were separated in a Petri dish, and lateral ventricles were located, opened and the transparent membrane between the choroid plexus anteriorly and striatum posteriorly was harvested. Harvested tissues were dissociated chemically using a papain dissociation kit (Worthington Biochemical Corporation, Lakewood, NJ, USA) according to the company's instructions while shaking mechanically on a rocker to facilitate cells dissociation. After complete dissociation, cells were separated using a density gradient centrifugation at 1500 rpm for 5 min. aNSPCs were expanded as neurospheres in a chemically defined serum-free growth medium. Cells were counted and passaged every week and low passage number (3–5) cells were used for encapsulation into the hydrogel.

2.3 aNSPC Encapsulation in MAC Hydrogels

The first step in the assembly of the scaffolds was to dissolve lyophilized MAC in ultrapure water at a concentration of 3 wt%. A stock of photo-initiator of 1-hydroxycyclohexyl phenyl ketone (IRG-184; Sigma-Aldrich, St. Louis, MO, USA) at 300 mg/mL in 1-vinyl-2-pyrrolidinone (Sigma-Aldrich, St. Louis, MO, USA), was prepared and filtered through a 0.2 mm syringe filter. Next, photoreactive laminin at a concentration of 50 mg/g, 3 million cells per gram material, and 14.7 mM IRG-184 were added to the MAC solution. To attain 2 wt. % buffered MAC solution, 10× PBS was added to reach the final desired volume. When all components were added, this resulting mixture was gently mixed (0.5–1 min, 1500 rpm, SpeedMixer DAC 150 FVZ, Hauschild Engineering, Hamm, Germany) and then 150–200 µL of the mixture was transferred into a 96-well plate. Finally, free radical polymerization was achieved by exposure to UV (365 nm, 2.7 mW/cm²) light for about 3 min to form hydrogels. Scaffolds were used for RNA extraction immediately after synthesis.

2.4 RNA extraction and analyses

RNA extraction began using mechanical disruption and homogenization of the encapsulated cells in the corresponding lysis buffer of one of three extraction methods explained below. Before the process of disruption and homogenization, pH of lysis solution was checked using Litmus paper (range 0–13) and adjusted to the desired pH using NaOH (1–5 M) and HCL (1–5 M) using a dropwise addition. For disruption of the scaffolds, a polypropylene pellet pestle was used, followed by homogenization using a syringe and needles of gradually decreasing size.

The RNeasy Plus mini kit (Qiagen, Valencia, CA) was used to extract RNA from aNSPCs encapsulated in MAC hydrogel according to the manufacturer's instructions. Genomic DNA was removed by the gDNA Eliminator spin column accompanying the kit.

TRIzol reagent (Ambion, Foster City, CA) is a mixture of phenol, guanidine isothiocyanate, and red dye. 1 mL TRIzol reagent was added to scaffold samples which were homogenized as previously mentioned. 200 µl chloroform (molecular grade, Sigma-Aldrich) was used to extract the RNA and spun down at 12,000 g for 15 min at 4 OC. The aqueous phase was precipitated with isopropanol (molecular grade, Sigma-Aldrich) for 10 min and centrifuged at 12,000 g for 10 min at 4 OC. 75% ethanol (molecular grade, Sigma-Aldrich) was used to wash the RNA twice, centrifuging at 7500 g for 5 min at 4 OC, in between. Pellets were air-dried for 20 min and resuspended in PCR-grade water.

Finally CTAB buffer, it is a freshly-prepared mixture of cetyltrimethylammonium bromide (CTAB), polyvinylpyrrolidone, NaCl, TRIS-HCl, and EDTA [19]. All these components were mixed and autoclaved, then supplemented with 1% β-mercaptoethanol. 600 µL of the pre-warmed extraction buffer was used to homogenize each scaffold, as described above. Following mixing of the homogenate, an equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed well, then centrifuged for 5 min at 15,000 X g at RT. The clear upper phase was transferred to a new tube and mixed with an equal volume of isopropanol before centrifuging it for 15 min at 15,000 g at RT. After discarding the supernatant, the pellet was washed in 1 mL of 75% ethanol. Finally, the pellet was dissolved in 40 µL RNase-free water.

Following RNA extraction, RNA concentration and purity of all samples were measured using a UV spectrophotometer (Infinite M200 with NanoQuant Plate, Tecan, Grödig, Austria), and the RNA integrity was evaluated using microgel electrophoresis via Agilent 2100 BioAnalyzer system (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions.

2.5 cDNA library creation

Two sets of cDNA libraries were made. For the first set, all sample concentrations were equilibrated to the same concentration such that all samples were diluted to match the lowest value. This allows for comparing different RNA samples of the same concentration and represents a comparison between the quality of the samples. The second set was made from RNA that was not equilibrated and served as a downstream teste of the yield of each sample. The synthesis of cDNA from each sample was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the company's protocol.

Anchored oligo (dT)18 primer was selected for the RT reaction. MS2 RNA was used to stabilize the template RNA due to the presence of low RNA concentration. cDNA was stored at -20°C and $2\text{ }\mu\text{L}$ was used for each qPCR reaction. Negative controls consisted of No Reverse Transcriptase (NRT) controls, where the reverse transcriptase enzyme was replaced with PCR-grade water.

2.6 qPCR

qPCR analysis was performed using LightCycler 480 SYBR Green I Master Mix (Roche, Basel, Switzerland) on a Roche LightCycler 480 II. The sequence of the forward and reverse primers of the three reference genes used are shown in Table 1. F and R primers concentrations ranged from 0.3 to $0.5\text{ }\mu\text{M}$ and were optimized prior to performing qPCR. qPCR was achieved by the following steps: 15 min activation (95°C), 15 s at 94°C , 15 s at 60°C , and 10 s at 72°C . Forty-five total cycles were performed. Three samples were measured in triplicate and the absolute values of the threshold cycles were used for comparisons.

2.7 Ionic interaction determination

To determine ionic interactions between methacrylated chitosan (MAC) and RNA in different pH conditions, zeta potential analysis was performed on MAC-RNA complexes versus controls using a zetasizer instrument (Zetasizer Nano S90, Malvern, UK) to match the neutral, acidic and basic pH conditions used in RNA isolation. To perform the zeta potential measurements, 1 wt% of MAC was used to prepare MAC only samples. RNA only samples isolated from aNSPCs had a concentration of $10\text{ ng}/\mu\text{L}$. MAC-RNA samples were also prepared with the same concentration of MAC and $10\text{ ng}/\mu\text{L}$ of RNA added together at a ratio of 1:1. Ultrapure water was used as the media for neutral pH. HCL (1 M) and NaOH (1 M) were used to adjust basic and acidic conditions, respectively. Samples were sonicated in an ultrasonic water bath (VWR, PA, USA) for 5 minutes with a power of 590 W before charge measurements. Pure MAC and RNA at neutral pH served as controls to compare to the complexes. To measure the zeta potential, standard folded capillary zeta cells with electrodes were used (Malvern). Zeta potential values were calculated using Smoluchowski relation [20].

2.8 Data analyses and statistics

Statistical analysis was performed using Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) software.) and JMP Pro 13. Data were analysed using ANOVA with Tukey's *post-hoc* test with $\alpha = 0.5$ to detect significance between different groups. When the comparison is between two groups only, student t-test was used to detect significance. All results are shown as mean $\pm$ standard error of the mean (SEM) unless otherwise indicated.

3. Results

3.1 Isolated RNA yields

The first set of analyses examined the impact of chitosan on the isolated RNA concentration and purity. The experimental group contained aNSPCs encapsulated in chitosan (Chitosan +ve), while the control group contained only aNSPCs without any scaffold material

(Chitosan -ve). The results obtained from this analysis are presented in Fig. 2. As expected, the presence of chitosan significantly impacted the concentration and A260/280 ratio of isolated RNA, when control and experimental groups are compared.

Further analysis showed that the methods were significantly different from each other in the control groups (no chitosan), $p < 0.05$. CTAB gave the highest concentration of RNA and RNeasy Mini kit gave the highest purity as expected. On the other hand, the experimental group, where cells are encapsulated in MAC, showed no significant difference between samples extracted using the three extraction methods with respect to RNA concentration and purity (p value > 0.05).

3.2 RNA assessment

The results obtained from the analysis of the interaction effects are shown in Fig. 3. From these data, positive control samples showed the highest values of RNA concentration along with acceptable purity values compared to all other pH values and methods. RNA samples isolated using TRIzol at different pH values showed high concentration, even though the purity varied at different pH values. On the other hand, samples isolated using the RNeasy kit showed consistently low concentrations, even though the purity ratio was acceptable. In the case of CTAB isolated samples, the purity, as indicated by the A260/280 ratio, improved as the pH value was increased. This trend was not the same when it came to concentration. The increase in A260/280 ratio in relation to increase in pH value was also observed with RNeasy kit isolation, however, in the latter case, the ratio did not go beyond the cut-off point even in the lowest pH.

3.3 RNA integrity is assessed by microgel electrophoresis

Fig. 4 provides representative electropherograms for samples from control and experimental groups with the concentration of the sample and its RNA Integrity Number (RIN) reported. RIN values ranged from 10 (intact RNA) to 1 (completely degraded RNA). As shown on Fig. 4, positive control samples (no chitosan) showed high RNA concentration. The highest RIN value was reported from samples isolated using the Qiagen kit, either experimental or control, when biological/assay pH was maintained as shown in Fig. 4A,E. CTAB performed better in basic pH (Fig. 4D), while the RNA integrity dropped at acidic pH (Fig. 4G). Fig. 4F, I, and H show the variability of TRIzol results. To combine the qualitative and quantitative results acquired, a selection matrix was put together, as shown in Table 2, to categorize the techniques and provide a quick guide for technique selection.

3.4 Gene expression analysis

For further assessment of the quality of recovered RNA, samples of the total RNA were analysed by RT-qPCR. The mean of the threshold cycles (C_t) from three samples was compared in respect to three different reference genes as shown in Fig. 5. Fig. 5B and 5B show comparisons between the normalized and non-normalized RNA. This reflects a comparison between the yield of different samples. Finally, genomic DNA contamination of samples was assessed through amplification of No Reverse Transcriptase (NRT) controls where RT was replaced by PCR-grade water during the RT reaction, No Template Control

was also used to test for qPCR contamination and both controls showed no amplification (results are not shown).

3.5 Zeta potential measurements

The results of zeta potential measurements on MAC-RNA complexes at different pH conditions are shown in Fig. 6. These data show variations in charge density of the colloidal dispersions as a response to the acidity of the surrounding media. The presence of the cationic amine groups in methacrylated chitosan structure as well as the anionic phosphate groups in RNA backbone, enables these two polymers to form polyelectrolyte complexes in certain conditions. Zeta potential is calculated based on the electrophoretic mobility of the polyelectrolyte complex domains in the system, which is then converted to the electrical charge density [21]. At neutral pH (Fig. 6A), the charge density has lowered when MAC was complexed with RNA. At neutral pH, the zeta potential value for the RNA only control was -26.63 ± 0.99 mV. However, in acidic conditions (pH = 2, Fig. 6B), there was an increasing trend in the charge density of the MAC-alone sample as compared to when it was complexed with RNA. At the basic pH condition (Fig. 6C), the pH of the colloidal system resulted in a zeta value of -6.67 ± 0.2 mV for the MAC-only sample and -4.80 ± 0.8 mV for the MAC-RNA complex. The decreasing trend of charge density of MAC-RNA compared to MAC in basic pH is similar to what was seen at neutral pH. Overall, these results suggest that there are electrostatic interactions between the cationic amine groups of MAC and the phosphate groups present in RNA chains, which change with the overall pH conditions.

4. Discussion

For RNA isolation to accurately reflect cellular transcriptional responses to their environment, sampled and isolated RNA should be abundant enough, high quality and with minimal contamination. Polysaccharides are widely used polymers in TE and drug delivery applications due to their encouraging properties such as compatibility, degradability, and ability to be modified/functionalized. As mentioned in the introduction, chitosan is a widely studied polysaccharide that causes entrapment and contamination of RNA isolated from cells encapsulated within it. Previous studies evaluating RNA extraction from cells encapsulated within chitosan observed inconsistent and low yields with various extraction methods due to electrostatic interactions between RNA and the polymer [8]. Therefore, optimization of the most commonly used methods of RNA isolation has been attempted by researchers in the past, yet none of these reports have investigated if pH of isolation reagents affects yield and purity of RNA isolated from chitosan, even though it has been previously reported that pH impacts the overall charge of RNA [22] and chitosan in solution [23,24], suggesting that RNA binding and release from chitosan is pH dependent. Moreover, this change of pH can even impact the hydrogelation process of chitosan according to Wahid et al.[25].

The results of this study confirmed that the presence of chitosan impacts the RNA extraction process using any of the three common extraction methods as shown in Fig. 2. This finding broadly supports the work of other studies [26–28] linking natural polymers with positively charged amine groups with decreases in RNA yield or quality. Several possible explanations

could describe these findings. The low yield of isolated RNA is thought to be due to ionic complexation between the negatively charged RNA and positively charged regions of the biomaterial matrix. This explanation is experimentally supported by a study showing that positively charged polymer hydrogels interfere with RNA extraction because they electrostatically interact with each other [26]. Moreover, other studies illustrated that positively charged chitosan forms ionic complexation with the drug Doxorubicin hindering its release [29,30]. Furthermore, we provide new data to show that the zeta potential of a chitosan solution at neutral pH is lowered after mixing it with RNA (Fig. 6). Importantly, this interaction also impacts the quality of isolated RNA. In addition to ionic interactions, physical interactions occur where RNA is adsorbed to chitosan macromolecules [31,32]. According to a homopolymer adsorption model by Melzak *et al.* [33], RNA may adsorb to about 70% of the surface area of the sorbent surface.

Different methods of total RNA extraction were compared in this study. As expected, with the cell-only group, filter-based spin columns showed the highest purity and CTAB showed the highest concentration as there was no interference of chitosan to the extraction process. When chitosan is included, the differences between method did not show a statistical significance at regular pH ($p < 0.05$, Fig. 2). However, CTAB shows the highest purity, while TRIzol yielded the highest concentration (Fig. 3). These findings are supported by the findings of Yu *et al.* [34], who discuss that the pH of CTAB (about 8) is the reason behind the ability of this method to remove the polysaccharide by minimizing the interaction between the positively charged chitosan fragments and the negatively charged RNA. TRIzol concentration results can also be explained by the very low pH, while the phenol component of TRIzol is a known contaminate for downstream applications that often results in low purity results. Our results agree with another study that needed a second method of RNA cleaning or purification with TRIzol to increase RNA purity [34].

To overcome the impact of chitosan presence in samples for RNA isolation, different strategies have been applied previously. Notably, modified forms of the CTAB method have been used to extract RNA from difficult plant tissues containing abundant levels of polysaccharides [32,35]. Researchers have also combined methods, to better reap the benefits of each method [27,34]. However, these modifications and combinations have rendered the process complex and sometimes prone to contamination, while also reducing overall yields. As an alternative to complex combinations of methods, chitosan digestion through lysozyme or chitinase seems like a reasonable step to reduce chitosan and RNA interactions [8,34]. The typical procedure exposes the scaffolds containing live cells to digestion enzymes for 24 – 48 h, however, the limitation with this is it more than likely triggers the cells to change their gene expression profiles, leading to inaccurate results. Yu *et al.* (2013) tried to overcome this limitation by snap freezing hydrogel samples in liquid nitrogen immediately after fabrication, then repeating snap-freezing after digestion. This approach improved quality but led to the lowest overall total RNA yield of the approaches studied. Even though, snap-freezing is a common approach to slow down the gene expression machinery, it may cause RNA degradation due to the disruption of cellular components where RNase is sequestered giving RNase access to RNA.

To the best of our knowledge, this work details the first study assessing the impact of pH manipulation on the extraction efficiency and quality of RNA from cells encapsulated in a chitosan-based material. Our findings are also supported by a previous study that used charged self-assembled peptides and postulated a role for pH during isolation [26], however, in this report pH was not directly studied and enzymatic pre-digestion was employed. Amongst the methods we tested, TRIzol at pH 5 showed the highest concentration different from all other reagents while giving the lowest purity value. Altogether, this high total RNA concentration may be due to the absorbance by contaminants [36]. High and low pH values both helped TRIzol and CTAB to give higher concentrations. However, purity was impacted differently, but logically (Fig. 3) as more alkaline pH improved the purity values for both methods, while an acidic environment improved the purity of samples isolated with TRIzol, but not CTAB. These results could be explained by the fact that these pH values correspond with the acidic (pH 2) and alkaline (pH 8) nature of TRIzol and CTAB, respectively. Moreover, in another study, researchers studied RNA binding and release from chitosan-coated silica particles, they found that chitosan releases RNA quickly at pH 9, while it binds to it at pH 5 [16]. Our findings are contrary to a previous study [37], which suggested that isolating under basic pH may be disadvantageous in removing genomic DNA contamination as the polar DNA and RNA will both partition into the aqueous phase. However, as it has been discussed earlier, the presence of chitosan changes the dynamics of isolation due to change in electrostatic forces in the homogenate. Moreover, this result may be explained by the finding of another study [38] that highly deacetylated chitosan releases DNA very slowly, which may suggest in our study that genomic DNA precipitated with chitosan to counteract the effect of high pH during extraction. The effect of solution pH on the release of small molecules from chitosan hydrogels is also supported by drug release studies indicating that physiological pH leads to the formation of ionic complexes between chitosan and specific model drugs such as curcumin [39] and doxorubicin [40] in addition to drug entrapment within the hydrogel [25,29,39,40].

Turning to our downstream application testing using qPCR. At conventional pH of the extraction method, control samples resulted in lower Ct values constantly which indicates higher quality of reverse-transcribed RNA (Fig. 5). Normalized RNA employs a constant amount of total RNA in each reaction. As such, the Ct values rely on, other than reaction conditions, the quality of RNA. The non-normalized RNA utilizes a constant volume of total RNA solution in each reaction, and because the yield of isolated RNA varies, the amount of RNA in each reaction was different. However, the observed differences between samples did not allow drawing solid conclusions. One notable trend is that the quality of RNA isolated using the Qiagen filter-based kits is superior, while non-normalized samples show higher Ct values, which is indicative of low initial concentrations (Fig. 5B). Still, samples isolated using CTAB at pH 9 always show low Ct values, which indicative of higher quality (Fig. 5B). There are several previous studies that have isolated RNA from cells encapsulated in chitosan [8,34,41], amongst others. The discrepancies of these studies may be explained by differences in the size of the construct and the number of cells within each construct, or the number of protonated amine groups and degree of deacetylation. Other possible causes include the degree of crosslinking and whether the cells in matrices were frozen before extracting RNA.

Ionic interactions have been previously implicated to affect RNA extraction from chitosan-based biomaterials [8,34]. As a result of these ionic interactions, charge neutralization likely occurs in the chitosan backbone after complexing with RNA anions, which results in lowering the overall electrical charge of the system [41] (Fig. 6A). The charge density of colloid systems is an important factor to consider in determining the degree of complexation as well as stability [42,43]. The extent of this complexation corresponds to zeta potential variations in the colloidal dispersion [41]. At neutral pH the charge density lowered by ~15% when MAC was complexed with RNA (Fig. 6A), and is likely due to the neutralization of the positive charges of the amino groups (NH_3^+) of MAC via the anionic phosphate groups (PO_4^{3-}) of RNA [41,44]. However, this trend was reversed in acidic conditions ($\text{pH} = 2$), which can be explained by two competing phenomena. First, the selected acidic conditions cause the amino groups of MAC to be highly protonated with a high density of H_3O^+ charges surround it. It is believed that these conditions cause chitosan chains to salt out and precipitate as non-dissolved polymers to reduce the concentration of chitosan in solution [45–47]. Second, the selected acidic conditions also facilitates hydrolytic degradation of the glycosidic bonds in the chitosan backbone, resulting in polymer degradation [48,49], which results in improved release [39]. Both salting out and hydrolytic degradation phenomena likely correspond to the lower zeta potential value observed in the MAC samples in $\text{pH} = 2$ compared to neutral pH (Fig. 6). When complexed with RNA though, the positive charge density of the environment and/or MAC is neutralized via the phosphate groups of RNA resulting in less degradation and precipitation of chitosan, as confirmed by higher zeta potential values (Fig. 6B). Chitosan is typically solubilized using dilute acidic conditions [50]. Therefore, the solubility of MAC in $\text{pH} = 9$ is limited [51], resulting in very low zeta potential values observed for the samples in basic conditions further indicating their instability (Fig. 6C) [52]. Watson et al. [53] have also confirmed the instability of similar polyelectrolyte complexes when the zeta potential is lower than 30 mV. Thus, the zeta potential measured in $\text{pH} = 9$ is likely primarily due to the solvent.

5. Conclusions

The main goal of this study was to apply a pH-dependent approach for the extraction of high quality and purity RNA from cells encapsulated in chitosan-based biomaterials. These findings will be of interest to researchers who use chitosan and other charged polymers as a platform for their TE and regenerative medicine approaches and need to probe transcriptional responses. The results demonstrated that manipulating pH of the scaffold homogenate to values of 5 and 9 influences the concentration, quality, and integrity of isolated RNA, however, this effect depends on the method used and the starting pH of the homogenate. High pH conditions result in high yields with good quality using both TRIzol and CTAB. pH of the homogenate did not affect RNeasy spin columns, which works best in neutral conditions with good quality, however, the overall yield was low. Considering the opposite electrical charges present in chitosan and RNA backbones, we used zeta potential as a means to demonstrate the extent of polyelectrolyte complex formation between these two polymers. This supported the selected pH conditions as a target to alter these ionic interactions during RNA extraction to maximize RNA yield.

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Highlights

- A pH-based approach for RNA isolation from chitosan-encapsulated cells is proposed.
- Manipulating pH during homogenization improve yield and purity of isolated RNA.
- Commonly used methods of RNA extraction work differently at different pH values.
- Ionic interaction between RNA and chitosan is experimentally proven.

Figure 1.
Synthesis of MAC for the generation of neurogenic construct (structures shown for chitosan and MAC are repeat units).

Figure 2.

Chitosan affects the RNA yield and quality. Spectrophotometric comparisons between experimental group (NSCs encapsulated in chitosan-based hydrogel) and control group (cells without chitosan hydrogel). A) shows the RNA concentration where the control group show significantly higher concentrations than the experimental group. Within the experimental group there was no statistically significant differences when different extraction methods were compared ($p=0.63$). Within the control group higher RNA concentrations were obtained using the CTAB method. Asterisks represent significant difference by Two-way ANOVA (* denotes $p<0.05$, *** denotes $p\text{ value} < 0.001$) and *post-hoc* analysis, data is presented as Mean $\pm$ SEM with $n = 5$.

Figure 3.

At different pH, extraction methods yield different RNA concentrations of different quality when different pH values are compared to the conventional pH of the extraction method. Spectrophotometric comparisons of A) RNA concentrations isolated using RNeasy Midi Kit, CTAB, and TRIzol at different pH are yielding different concentrations. The yield of the control samples (measured at the conventional pH of each extraction method) was always higher than its corresponding experimental samples, as well as the B) purity of isolated RNA measured by 260/280 ratio. Dotted line indicates the cut-off point for pure RNA and differences in number above it doesn't reflect difference in purity. Statistical analysis indicated significant difference by Two-way ANOVA followed by post-hoc test. Letters and asterisks indicate difference. Bars not connected by the same letter are significantly different and *** denotes $p < 0.001$. Data are presented as Mean $\pm$ SEM, $n=5$.

Figure 4.

The quality and yield of samples are differentially impacted by extraction method and pH. Even with high quality, the yield might not be sufficient. A – C) show examples of +ve control samples (aNSPCs with no chitosan) of the three methods used where both yield and integrity of RNA are of high quality. D-E) representative results of Agilent Bioanalyzer of samples of high yield and adequate integrity and samples with enough yield and high integrity. F-H) represent samples with mixed results of yield and integrity. I-J) represent results with poor yield and integrity. K) is a ladder image.

Figure 5.

Effect of pH and extraction method on downstream qPCR application. A) shows a comparison between experimental and control groups. RT-qPCR results of B) non-normalized (RNA amount as it was extracted, and it reflects RNA quantity comparison) and C) normalized (where are RNA samples are diluted to the same concentration and this reflects RNA quality comparison) extracted RNA using three references genes; 18S, GAPDH, and HPRT. Results are shown as Ct values (lower is more abundant). Statistical analysis shows the effect of the interaction between extraction method and pH on downstream applications. The statistics for comparing exp vs CTRL groups used a student's t-test and the rest used One-way ANOVA (letters denote significance ($p < 0.05$)). Mean $\pm$ SEM, $n = 5$, 3 for normalized groups.

Figure 6.

Zeta potential analysis of MAC and MAC-RNA complex showing variations in surface charge in different pH conditions due to polyelectrolyte interactions. (A) Comparison at neutral pH; (B) Acidic pH = 2; (C) Basic pH = 9. Data all n = 3, mean $\pm$ SEM. Asterisks denote significance as determined by pairwise t-tests with * showing $p < 0.05$, and *** $p < 0.001$.

Table 1.

RT-qPCR primer sequences

Primer name	Forward Sequence	Reverse Sequence
HPRT	5'-CTCATGGACTGATTATGGACAGGA-3'	5'-GCAGGTCAGCAAAGAACTTATAGC-3'
GAPDH	5'-AGCTCATTTCTGGTATGACAA-3'	5'-TACTCCTTGGAGGCCATGTA-3'
18S	5'-GATTAAGTCCCTGCCCTTTG-3'	5'-CCTCACTAAACCATCCAATCG-3'

Table 2.

Selection matrix of RNA extraction method and homogenate pH based on yield and purity of extracted RNA

Concentration (ng/μL)	Purity (A260/280)			
		Low (<1.7)	Medium (1.7 < * < 1.8)	Hight (>1.8)
	Low (<10)	RNeasy, pH 9	RNeasy, pH 5	
	Medium (10 – 25)		CTAB, pH 7	RNeasy, pH 7
	High (>25)	CTAB, pH 5 TRIzol, pH 5	TRIzol, pH 7	CTAB, pH 9 TRIzol, pH 2 TRIzol, pH 9