Published in final edited form as:

Biomaterials. 2010 March ; 31(7): 1612. doi:10.1016/j.biomaterials.2009.11.024.

Extraction of High Quality RNA from Polysaccharide Matrices using Cetyltrimethylammonium Bromide

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Abstract

Polysaccharides are increasingly being used as biomaterials for tissue engineering and regenerative medicine. Quantitative analysis of gene expression from cells in three-dimensional (3D) scaffolds requires extraction of messenger RNA, which is complicated in polysaccharide materials by ionic complexing between nucleic acids and the matrix. We used a strongly cationic surfactant, cetyltrimethylammonium bromide (CTAB), to extract RNA from human mesenchymal stem cells embedded in 3D chitosan, agarose and collagen matrices. CTAB extraction was compared to conventional guanidinium thiocyanate-based methods for RNA isolation by assessing RNA yield, purity (A260/A280 and A260/A230) and integrity (28S/18S and RIN). For polysaccharide-based matrices, CTAB extraction yielded significantly more RNA with higher purity than guanidinium thiocyanate-based methods alone. The extracted RNA was largely intact as indicated by 28S/18S ratios and RIN values, while these parameters could not be measured using conventional kits alone. For pure collagen matrices, the CTAB method was comparable or better than guanidinium thiocyanate-based methods in terms of RNA yield and quality. We further validated the CTAB protocol using semi-quantitative and quantitative RT-PCR to amplify both large and small amplicons. Our results show that the CTAB-based method is a facile and effective way to extract abundant, high quality RNA from polysaccharide and protein biomaterials.

Keywords

RNA; PCR; polysaccharide; tissue engineering; chitosan; collagen

1. Introduction

The extraction of messenger ribonucleic acid (RNA) from biomaterials used in tissue engineering and regenerative medicine is a common way to assess levels of gene expression, using techniques such as northern blotting, reverse transcription polymerase chain reaction (RT-PCR), and microarray analysis. Traditional methods of harvesting RNA from cells grown in two-dimensional monolayer culture have been successfully extended to three-dimensional (3D) scaffolds made of both synthetic polymer- and protein-based materials.[1,2] More recently polysaccharide-based materials have been used as scaffolds for cells, with an increasing use of agarose, alginate, chitosan, and other related sugar polymers in a variety of

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applications.[3] However, the use of 3D polysaccharide-rich matrices presents a problem for the acquisition of sufficient high-quality RNA for robust biological evaluation, since these materials can interfere with commonly used RNA extraction techniques.

Most common commercial RNA isolation kits are based on the use of an acid guanidinium thiocyanate-phenol-chloroform extraction reagent, as pioneered by Chomczynski and Sacchi.[4] The widely used TRIzol® family of reagents (Invitrogen, Carlsbad CA) is based on this technology. A basic TRIzol® procedure includes tissue digestion in a chaotropic lysis buffer, the separation of a RNA-containing aqueous phase from organic phases, and subsequent RNA precipitation. Large polysaccharide fragments present after sample digestion can entrap nucleic acids physically and be entrained during centrifugation into the discarded phase during phase separation, thus leading to a low yield.[5] Moreover, small polysaccharide fragments can partition into the aqueous phase during phase separation and co-precipitate with RNA in the RNA precipitation step, which interferes with downstream applications.[6,7] Another widely used RNA isolation product, the Qiagen RNeasy kit also uses a guanidinium thiocyanate buffer for sample lysis, and uses a silica membrane spin column to purify the RNA. However, the existence of polysaccharide contamination has been shown to decrease the efficiency of spin-columns significantly.[8]

The present study was motivated by the need for better methods to extract sufficient quantities of high quality RNA from polysaccharide matrices for use in gene expression assays. We examined the use of an extraction buffer based on a cationic surfactant, cetyl trimethylammonium bromide (CTAB), to isolate nucleic acids from both protein- and polysaccharide-based hydrogels containing embedded cells. The use of CTAB to extract nucleic acids originated in the 1950s with bacterial samples, [9] and later was also used to isolate DNA and RNA from plant tissues containing a high level of phenolic compounds and polysaccharides.[8,10,11] Like guanidinium thiocyanate-based procedures, a CTAB protocol includes an initial nucleic acid extraction step, a phase separation step using chloroform, and a precipitation step to separate RNA from DNA and polysaccharide residues. A typical CTAB extraction buffer contains CTAB, polyvinylpyrrolidone (PVP), sodium chloride, and beta-mercaptoethanol, each of which plays an important role in nucleic acid extraction from polysaccharides. CTAB acts as a strong detergent to help to break (plant) cell walls and separate nucleic acids from polysaccharides.[12] Salts help to dissolve polysaccharides and CTAB-RNA complexes, resulting in the efficient removal of polysaccharides and CTAB during chloroform extraction.[13,14] PVP helps to prevent the oxidation of polyphenols in cell walls and extracellular matrices.[13] Beta-mercaptoethanol is a strong reducing reagent that acts to irreversibly denature RNases.

CTAB-based methods have been used increasingly for nucleic acid isolation from polysaccharide-rich plants, and in particular RNA isolation in recent years.[5,7,8,10,15–18] In contrast, CTAB-based methods have not been widely employed with animal and human cells, perhaps due to their relatively low polysaccharide and polyphenol contents. Here we report the application of a CTAB-based method for RNA extraction from adult human mesenchymal stem cells (hMSC) that have been embedded and cultured directly in both polysaccharide (agarose, chitosan) and protein (collagen) hydrogel matrices. We isolated nucleic acids from hMSC using either a TRIzol® kit, a Qiagen kit, or using CTAB extraction buffer followed by purification on a Qiagen spin-column. We also examined the combination of TRIzol® extraction followed by purification on a Qiagen spin-column as reported previously.[19] RNA yield, purity (A260/A280 and A260/A230) and integrity (28S/18S and RIN) were assessed for each sample and extraction type. In addition, isolated RNA was assessed for use in PCR assays.

2. Materials and Methods

2.1 Fabrication of Cell-Seeded Polysaccharide and Protein Hydrogels

hMSC at passage 1 were purchased from Lonza Inc. (Walkersville MD) and cultured in a medium consisting of Dulbecco's Modified Eagle Medium (DMEM, low glucose formulation), 10% MSC-qualified fetal bovine serum (Invitrogen, Carlsbad CA), and 1% penicillin/streptomycin (Invitrogen). hMSCs were expanded to passage 4 and then seeded in the three types of gels. Chitosan matrices were prepared by combining cold 2.0 % (wt/vol) chitosan (93% DDA; BioSyntech Inc., Quebec, Canada) dissolved in 0.1 N acetic acid with cold 0.04% (wt/vol) collagen Type I (MP Biomedicals, Solon OH) dissolved in 0.02 N acetic acid at a mass ratio of 65:35. hMSC were added at a concentration of 2.0×10^6 cells/ml and the gelling process was initiated by the addition of 10.0 % (wt/vol) beta-glycerophosphate (Sigma Chemical, St. Louis MO). Constructs were incubated at 37 °C for 30 min until gelation was complete and then were transferred to culture medium. The reason for the addition of a small amount of collagen to the chitosan matrices was to promote cell attachment.

Agarose matrices were prepared by suspending hMSC at 1.0×10^7 cells/ml in melted 2.0 % (wt/vol) agarose (Sigma) at 37 °C and then initiating gelation by rapidly dropping the temperature to 4 °C for 20 min. Fully gelled constructs were then transferred to culture medium at 37 °C.

Pure collagen gels were prepared by adding hBSCC to cold solution of 4.0 mg/ml collagen Type I (MP Biomedicals, Solon OH) dissolved in 0.02 N acetic acid. Gelation was initiated by adding 1.0 N sodium hydroxide and warm 10X concentrated PBS. The resulting solution was used to re-suspend cells and gelled at 37 °C for 30 min. Constructs were then transferred to culture medium.

Chitosan and pure collagen matrices were cultured for 1–3 weeks prior to analysis, and agarose samples were used on the day of fabrication in order to ensure survival of the embedded cells. All samples were snap-frozen at liquid nitrogen (LN₂) and stored at –80 °C for subsequent use in RNA extraction studies.

2.2 Preparation of CTAB Buffer

The CTAB extraction buffer was modified from Chang *et al.*[14] and was composed of 2.0 % CTAB (Sigma), 2.0 % polyvinylpyrrolidone (PVP 40; Sigma), 1.4 M sodium chloride (NaCl, Sigma), 100 mM Tris-HCl (pH 8.0; Sigma), 20 mM ethylenediaminetetraacetic acid (EDTA; Sigma), and 1.0 % beta-mercaptoethanol (Sigma) in RNase-free water (Qiagen Inc., Valencia, CA). This buffer was used for chitosan and collagen samples and this buffer was supplemented to 2.0 M NaCl when used for agarose samples. The components in the extraction buffer, excluding beta-mercaptoethanol, were mixed, autoclaved, and then supplemented with 1% beta-mercaptoethanol (Sigma) before RNA extraction. The final solution was warmed in a dry bath to 65 °C for use in RNA extraction studies.

2.3 RNA Extraction

Samples at –80 °C were crushed into fine powders in a BioPulverizer (BioSpec Products Inc., Bartlesville OK) which was pre-cooled in LN₂. These powders were used for the following extraction protocols including the CTAB method, TRIzol® kits, Qiagen Kits, and their combination.

For the CTAB method, sample powders were transferred to RNase-free microcentrifuge tubes filled with 600 µl pre-warmed CTAB extraction buffer and vortexed for 5 min at room temperature. Following mixing, an equal volume of chloroform-isoamyl alcohol (24:1) (Fisher

Scientific, Pittsburgh PA) was added, mixed well, and centrifuged for 5 min at 15,000 ×g at room temperature. After centrifugation, the clear upper phase was again extracted with an equal volume of chloroform-isoamyl alcohol. The upper phase was then mixed with an equal amount of isopropanol (IPA) and centrifuged for 15 min at 15,000 ×g at room temperature. The supernatant was discarded, and the pellet was washed in 1.0 ml of 75% ethanol and centrifuged at 15,000 ×g. The washed pellets were dissolved in 30–50 l RNase-free water and used for RNA purification following the manufacturer's protocol for the Qiagen RNeasy Mini kit, and the resulting RNA was stored at –80 °C.

For TRIzol® kits alone, a method modified specifically for polysaccharide-rich samples was used to extract RNA.[20] Briefly, after sample digestion in TRIzol®, the aqueous phase containing RNA was separated from the interphase and the organic phase by the addition of chloroform. In the following RNA precipitation step, the modified protocol used 0.25 ml IPA and 0.25 ml high-salt solution (0.8 M sodium citrate and 1.2 M NaCl) instead of 0.5 ml IPA. The RNA pellets were then washed in 75% ethanol in RNase-free water. Finally, the pellets were air-dried for 10 min, re-dissolved in 30–50 µl RNase-free water, and stored at –80 °C.

For Qiagen kits alone, sample powders were subjected to RNA extraction following the manufacturer's procedure. In brief, samples were dissolved in Qiagen lysis buffer and then homogenized in a QIAshredder homogenizer (Qiagen). Subsequently, the supernatant was mixed with 0.5 volume of 100% ethanol. The mixture was then transferred to RNeasy spin columns followed by one wash with RW1 buffer and two washes with RPE buffer. Total RNA was eluted with 30–50 µl RNase-free water and stored at –80 °C.

For the combination of the TRIzol® and Qiagen methods, the protocol developed by Wang et al was used.[19] Briefly, after phase separation using TRIzol® reagents, the aqueous phase was mixed with 0.5 volume of 100% ethanol and then applied to the RNeasy spin columns followed by purification according to the manufacturer's protocol, and the resulting RNA was stored at –80 °C.

2.4 RNA Assessment (Yield, Purity and Integrity)

The absorbance of RNA in RNase-free water was evaluated at 230, 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE). RNA yield was calculated based on the Beer-Lambert Law, according to which the yield in µg/mL is 40X the absorbance at 260 nm (A₂₆₀). RNA purity was assessed by the ratios of A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀. RNA integrity was evaluated by the ratio of 28S/18S ribosomal RNA (rRNA) and the RNA integrity number (RIN) using an Agilent 2100 BioAnalyzer (Agilent Technologies, Foster City CA). This instrument uses automated microfluidics, capillary electrophoresis, and fluorescence to evaluate RNA integrity. Ideally, an intact human rRNA 28S/18S ratio is about 2.7 since 28S and 18S rRNAs are approximately 5.0 kb and 1.9 kb in size.[21] However, a ratio of around 2.0 is generally considered as an indicator of highly intact RNA.[21] The RIN is a related measure of RNA quality that is based on a larger portion of the electrophoretic trace. The BioAnalyzer automatically computes this parameter, and an ideal non-degraded RNA sample has a RIN of 10.

2.5 RT-PCR Analysis

For semi-quantitative RT-PCR, first-strand cDNA was synthesized using the Invitrogen Superscript III first-strand synthesis system, in which RT was performed by 10-min incubation at 25°C, 50-min incubation at 50°C, and 5-min incubation at 85°C. PCR reaction was conducted in a Bio-Rad C1000 thermal cycler following the instruction of Invitrogen Platinum PCR SuperMix. PCR amplification conditions were 35 cycles of 94°C for 30s, 55°C for 60s, and 72°C for 60s. The reaction amplicons were resolved by Agilent 2100 BioAnalyzer using DNA

chips. Two commonly-used endogenous genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-2-microglobulin (B2M), were tested using primers as followed: 5'-GGGCTGCTTTTAACTCTGGT-3' (forward, GAPDH), [22] 5'-TGGCAGGTTTTTCTAGACGG-3' (reverse, GAPDH), [22] 5'-CTCGCGCTACTCTCTCTTTCTGG-3' (forward, B2M), [23] and 5'-GCTTACATGTCTCGATCCCACTTAA-3' (reverse, B2M). [23] For quantitative RT-PCR, messenger RNA (mRNA) was converted to cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems) following the supplier's procedure. TaqMan gene expression assay kits (Applied Biosystems) were used for transcript levels of GAPDH, type I collagen (CI), runt-related transcription factor 2 (RUNX2), SRY-box containing gene 9 (SOX9), and Myogenin (myogenic factor 4) (MYOG) in an Applied Biosystems 7500 Fast System. The kits were Hs99999905_m1 for GAPDH, Hs00164004_m1 for CI, Hs00231692_m1 for RUNX2, Hs00165814_m1 for SOX9 and Hs00231167_m1 for MYOG.

3. Results and Discussion

The CTAB-based method we used was adapted from methods used for plant cell biology. Our reasoning was that this method may work well for polysaccharide-based matrices in tissue engineering, since many plant tissues also have high polysaccharide content. [15] Plant cells have rigid cell walls that need to be degraded in order to expose internal nucleic acids to the following extraction. [24] Therefore, snap freezing in liquid nitrogen (LN₂) and grinding using mortars/pestles or pulverizer are commonly utilized to facilitate the breakage of plant cell walls. We also used these methods, though mammalian cells have lipid cell membranes which are more easily compromised. The polysaccharide content of the plant samples also binds nucleic acids and makes it more difficult to isolate and purify RNA. However the CTAB method proved to be superior to guanidinium thiocyanate methods in terms of both RNA quantity and quality.

Figure 1 shows the RNA yield, purity, and integrity results from 3D chitosan-based samples containing hMSC. The CTAB method gave the highest RNA yield, generally isolating 4- to 7-fold more RNA than the other three methods assessed. The higher absolute yield of the CTAB method (> 3 µg/million cells) offered sufficient RNA for downstream applications, while other methods produced less than 1 µg/million cells. The RNA isolated by the CTAB method also exhibited good purity. RNA absorbs UV light maximally at 260 nm, whereas protein absorbs at 280 nm and other contaminants including carbohydrates, phenol, and aromatic compounds generally absorb around 230 nm. Therefore the A₂₆₀/A₂₈₀ and the A₂₆₀/A₂₃₀ ratios are often used to indicate RNA sample purity. Generally, samples with ratio values in the range of ~1.8 – 2.0 indicate high purity RNA. The A₂₆₀/A₂₈₀ value for the CTAB method (1.91 ± 0.11) was in the desired range of 1.8–2.0, and slightly lower than that for the TRIzol®+Qiagen and the Qiagen kit methods (2.01 ± 0.26 and 2.16 ± 0.12). This indicates that protein contamination was not a main issue for these samples. Importantly, the CTAB method gave a much better A₂₆₀/A₂₃₀ value (1.55 ± 0.40), compared to the other methods (all below 1.0), suggesting that CTAB samples were less contaminated by salts, phenol, or polysaccharides. RNA integrity also was assessed using an Agilent 2100 BioAnalyzer which provides the 28S/18S ratio and the RIN for RNA quality control. The RNA prepared by the CTAB method had a 28S/18S of 1.83 ± 0.45 , which is close to the ideal value of 2.0, and a RIN of 7.17 ± 0.91 , demonstrating that only modest RNA degradation occurred during the extraction process. In contrast, the integrity of the RNA from the other extraction methods could not be assessed (N/A) by the BioAnalyzer instrument due to low sample concentration or/and low purity.

Chitosan matrices have a large number of amine groups, which are protonated (positively charged) in acidic environments. [25] Both the TRIzol® and Qiagen extractions are performed in a weak acid environment, and therefore the positively charged chitosan not only physically entraps nucleic acids, but also may chemically bind to them, resulting in low RNA yields. In

contrast, the CTAB method is performed in a basic environment ($\text{pH} \approx 8$). It can be expected that under these conditions the formation of chitosan-nucleic acid complexes is prevented, resulting in improved RNA yield. Attempts have also been made to adjust guanidinium thiocyanate extraction buffers to higher pH in order to avoid protonation of chitosan and resulting sequestering of RNA [26], however the measurement of RNA purity ratios was not accomplished, perhaps due to contamination by polysaccharides. Instead a nuclease (RNase) protection assay was used for gene identification. However, RNase protection assays are expensive and time-consuming, although they allow a low concentration of mRNA to be detected. In another study, porous chitosan scaffolds pulverized in LN_2 were suspended in Tris buffer ($\text{pH} 8.5$) to allow RNA to diffuse out from the samples. [27] In this way, RNA was isolated and used for quantitative RT-PCR. However, this method did not work for the chitosan samples gelled with beta-GP used in the present study, perhaps due to differences in the type of chitosan or/and the number of amine groups in the matrices.

Figure 2 shows yield and purity data for RNA extracted from hMSC embedded in pure agarose matrices. In this case it was noted that severe co-precipitation was observed using 1.4 M NaCl in the purification step, though this effect was significantly alleviated by increasing NaCl concentration to 2.0 M (unpublished data). At this NaCl concentration, the CTAB method produced an RNA yield ($> 1 \mu\text{g}/\text{million cells}$) comparable with the combined TRIzol®+Qiagen method used by Wang *et al.* [19] for agarose samples. The TRIzol® kit and Qiagen kit alone produced less than $1 \mu\text{g}$ RNA/million cells. The sample purity was very good for all extraction methods, with little protein contamination evident ($\text{A}_{260}/\text{A}_{280}$). There was evidence of polysaccharide or other contamination ($\text{A}_{260}/\text{A}_{230}$), particularly in the TRIzol® alone samples. It appears that the Qiagen purification method using spin columns may have increased purity, though at the expense of decreased yields. In cases that require high yield or that have a high polysaccharide residue after the CTAB extraction, LiCl and CsCl precipitations may be helpful, though these methods are time consuming. These data highlight the fact that the RNA extraction protocol needs to be tailored to suit the particular polysaccharide being used in order to maximize both RNA yield and quality.

As a control, we also extracted total RNA from hMSC embedded in pure protein matrices. The results from pure collagen Type I samples are shown in Figure 3. The TRIzol® method gave the highest yield, though all three methods yielded abundant RNA (the TRIzol®+Qiagen method was not used in this series due to the absence of polysaccharides). The high RNA yield of the TRIzol® method ($9.61 \pm 1.12 \mu\text{g}/10^6 \text{ cells}$) was probably due to the fact that it uses an isopropyl alcohol precipitation. This method of purification yields a wide range of RNA types including rRNA, mRNA, transfer RNA (tRNA), and other low-molecular-weight (LMW) RNA, whereas the Qiagen spin columns do not bind to 5S/5.8S rRNA, tRNA, and other LWN RNA. These smaller RNA units typically comprise 15–20% of the total cellular RNA [28], and this population may explain the difference in yield. The CTAB method also yielded a large quantity of RNA ($7.82 \pm 0.42 \mu\text{g}/10^6 \text{ cells}$), indicating that this protocol also was highly efficient for these matrices. The purity and quality of the RNA isolated from pure collagen constructs by all methods was also good. The TRIzol® method showed slightly more protein contamination ($\text{A}_{260}/\text{A}_{280}$ of 1.75 ± 0.04) as well as evidence of increased polysaccharide and/or phenol contamination ($\text{A}_{260}/\text{A}_{230}$ of 0.89 ± 0.47). The 28S/18S ratio was also somewhat lower for the TRIzol® method, but the RIN was high and similar to the other methods. The CTAB method showed high and consistent purity ($\text{A}_{260}/\text{A}_{280}$ of 2.01 ± 0.03 and $\text{A}_{260}/\text{A}_{230}$ of 1.55 ± 0.26), as well as high RNA integrity (28S/18S of 1.80 ± 0.21 and RIN of 7.63 ± 0.42). Taken together, these results show that the CTAB method performs well on 3D protein-based samples, with results similar or better than other conventional methods of RNA extraction.

To further validate the quality of the RNA obtained from the CTAB and other methods, we performed RT-PCR on sample genes and analyzed the assay response. Semi-quantitative RT-PCR was used to amplify two commonly-used endogenous control genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 700 bp) and beta-2-microglobulin (B2M, 300 bp), from chitosan-based matrices. The results are shown in Figure 4 and demonstrate that these amplicons were successfully processed by PCR and that these relatively large fragments of mRNA were intact in the original sample. The RNA extracted by the CTAB-method was also successfully used for quantitative RT-PCR with smaller amplicons (~100bp), as shown in Figure 5. A selection of genes commonly assayed in hMSC was amplified using real time RT-PCR: GAPDH, collagen Type I, Runx2, SOX9, and MYOG. The amplification curves demonstrate that these smaller mRNA sequences were largely intact in the RNA samples, though the MYOG gene was in low abundance. It should be noted that amplification of genes from chitosan-based samples could not be performed on RNA extracted using the TRIzol® and Qiagen methods, due to the low yield and low quality of the RNA. Finally, real time RT-PCR was used to amplify selected transcripts extracted from pure collagen Type I constructs using the CTAB, TRIzol® and Qiagen methods. The cycle threshold for each sample type was compared, as shown in Figure 6, and suggested that there was no significant difference in the amplification efficiency between the RNA extraction methods.

5. Conclusions

These results show that extraction of RNA using CTAB allows the rapid isolation of large quantities of high quality RNA from both polysaccharide- and protein-based 3D matrices. For polysaccharide-based materials in particular, the CTAB method shows important improvements in RNA yield, purity and integrity. This method therefore enables the analysis of gene expression from cells embedded in 3D polysaccharide matrices, whereas this is not possible using conventional TRIzol® or Qiagen methods. The CTAB method provides a new approach to assessing cell function in a variety of tissue engineering matrices and scaffolds that contain significant polysaccharide content, and may have applications in other biomaterials contexts in which polysaccharide materials are important.

Acknowledgments

We are grateful to the staff at the University of Michigan Comprehensive Cancer Center (UMCCC) Affymetrix and Microarray Core Facility for performing RNA integrity evaluation. This project was supported in part by the National Institute of Arthritis and Musculoskeletal and Skin Diseases through grant R01-AR053231.

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Figure 1. Yield, purity, and integrity of total RNA isolated from chitosan-based samples using different extraction methods ($n \geq 4$). Error bars and numbers in parentheses indicate standard deviations.

Figure 2.

Yield, purity, and integrity of total RNA isolated from pure agarose samples using different extraction methods ($n \geq 4$). Error bars and numbers in parentheses indicate standard deviations.

Figure 3. Yield, purity, and integrity of total RNA isolated from pure collagen Type I samples using different extraction methods ($n \geq 4$). Error bars and numbers in parentheses indicate standard deviations.

Figure 4. Fluorescence intensity curves for amplicons of reverse transcriptase polymerase chain reaction (RT-PCR) of two commonly used endogenous control genes, GAPDH (A, ~700bp) and B2M (B, ~300bp), amplified from RNA extracted from hMSC embedded in chitosan-based samples using the CTAB method.

Figure 5.

Amplification curve of real time RT-PCR of a selected set of genes (~100 bp amplicons for GAPDH, Collagen Type I, Runx2, SOX9, and MYOG) from RNA extracted from hMSC embedded in chitosan-based samples using the CTAB method. Error bars indicate standard deviations.

Figure 6. Graph of cycle thresholds (Ct) of selected genes from RNA isolated using CTAB, Qiagen, and TRIzol® methods and amplified using real time RT-PCR (n = 4). Error bars indicate standard deviations.