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Short communication

Concurrent extraction of proteins and RNA from cell-laden hydrogel scaffold free of polysaccharide interference

Chunming Wang[a], Xin Li[b], Yongchang Yao[a], Dong-An Wang[a,*]

[a] Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, N1.3-B2-13, Singapore 637457, Republic of Singapore
[b] School of Biological Sciences, Nanyang Technological University, Singapore 637457, Republic of Singapore

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ABSTRACT

Concurrent extractions of proteins and RNA from cell-laden scaffolds would be of great help to biomaterialists and tissue engineers. Here we describe a procedure to extract proteins from the discard solution generated during the RNA isolation from polysaccharide-rich hydrogel scaffolds. This approach allows to obtain proteins and RNA from same sample while eliminating the polysaccharide interference.

1. Introduction

The explosive development of tissue engineering has shown great promise to the treatment of severe degenerative disorders, and attracts researchers engaged in various fields from polymer engineering to cell biology to orthopaedic surgery. In the practice of tissue engineering, polysaccharide hydrogels are widely used as scaffolds and cell-delivery vehicles, for accommodating therapeutic cells that are induced to differentiate into desired lineages or produce bioactive molecules [1,2]. At different time points, researchers investigate the functionalities and commitment of these cells by analysing their gene expression profiles. Common tests include reverse transcription polymerase chain reaction (RT-PCR) and Northern blotting—at transcriptional level; as well as Western blotting and mass spectroscopy—at protein scale. In a conventional way, total RNA and proteins are separately extracted from two or more parallel samples for such purposes. However, one can envisage that a method allowing concurrent harvest of RNA and proteins from the same construct would be much more efficient—to save samples and provide more comparable and consistent results. It especially benefits researchers when the polymer scaffolds are elaborately synthesised and/or the cell/tissue supply is limited, e.g., the cells are primarily derived from patients or knockout mice. Additionally, such a method enables the investigations of nucleic acid–protein interaction of cells within three-dimensional (3D) artificial microenvironments.

Concurrent extraction of nucleic acid and proteins from monolayer cells or patient material has been demonstrated elsewhere [3,4]. Nevertheless, it is far more difficult for cells encapsulated in 3D polysaccharide hydrogels, as the high content of carbohydrates would pose severe problems. For instance, the most commonly used guanidine thiocyanate-based assays (e.g., TRIzol®) have been established to simultaneously obtain nucleic acids and proteins from monolayer cells or mammalian tissues [3], but showed powerless when being applied to polysaccharide-based tissues [5,6]. Undesired carbohydrate components are often co-precipitated with desired total RNA and/or proteins, rendering the product pellets scarcely dissolvable. Downstream applications like RT-PCR or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are subsequently hindered. Accordingly, numerous efforts were devoted to obtain high-quality RNA [5,6] or proteins [7,8] from a variety of polysaccharide-rich tissues: either engineered tissue constructs or native plant specimen. These practices, albeit neither aiming for co-extraction nor dealing with engineering tissues, provide both theoretical feasibility as well as technical references. Next, we assumed that, since RNA is generally less stable than proteins and degrading readily, a conceivable co-extracting method should primarily fulfil RNA acquisition; only after high-quality RNA is successfully obtained, proteins are to be isolated from polysac-

* Corresponding author. Tel.: +65 6316 8890; fax: +65 6791 1761.
E-mail address: DAWang@ntu.edu.sg (D.-A. Wang).

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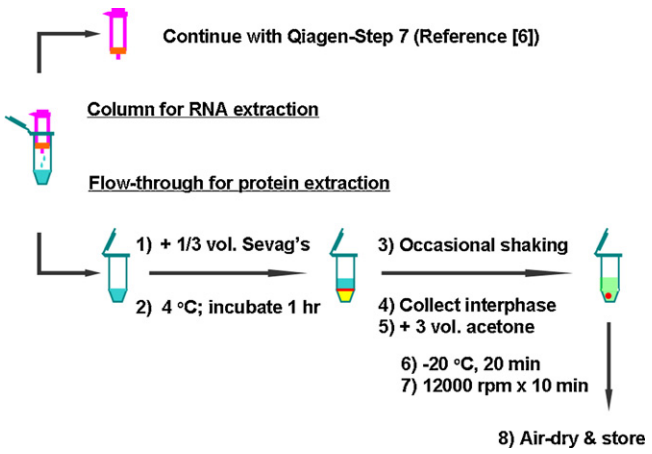

Fig. 1. Methodology of the concurrent extraction of RNA and proteins from polysaccharide-based hydrogels: after the centrifugation at Qiagen-Step 6, the spin-column was collected for RNA isolation, and the flow-through was retained and applied for protein extraction. After air-dry, protein pellets were either stored in −80 °C or subjected to SDS-PAGE electrophoresis.

charide contamination. Based on this consideration of priority, we set out the exploration with a commercially available kit, Qiagen® RNeasy Mini Plant Kit, which was originally designed for plant tissues but has proven reliable in isolating high-quality RNA from polysaccharide-based hydrogels [6]. By speculating that, during the RNA isolation, proteins (possibly with carbohydrates) are removed after Qiagen-Step 6s centrifugation, we attempted to recover proteins from this flow-through that is usually discarded.

2. Experimental

2.1. Cell-encapsulated hydrogel model

Human mesenchymal stem cells (hMSCs, Passage 2) were purchased from Cambrex (East Rutherford, NJ, USA), and expanded over multiple passages in mesenchymal stem cell growth medium (MSCGM, Cambrex). We chose MSC as model cell due to its wide application and promising potential in regenerative medicine [1,9]. The cells were re-suspended and encapsulated into agarose polysaccharide hydrogels (A-gel) at a density of 5×10^6/ml, and incubated in cultural medium on a shaker working at 50 rpm [9].

2.2. Protein isolation

Before protein isolation, one parallel construct was subjected to Live/Dead fluorescent indication (Invitrogen, Carlsbad, CA, USA) to confirm the viability of encapsulated cells. Then, samples were collected and frozen in liquid nitrogen for RNA isolation, following the procedure of Qiagen® RNeasy Mini Plant Kit till the completion of Step 6s centrifugation [6,10]. Then, as illustrated in Fig. 1, the spin-column was applied to continue the procedures of this kit specific for RNA isolation [6,10]. The flow-through (FlowTS-6), usually discarded, was retained for protein extraction. We applied 1 volume

Table 1
Proteins identified by Q-Star mass spectrometry against International Protein Index (IPI) human database.

Accession	Name
IPI:IPI00021439.1	Gene_Symbol = ACTB actin, cytoplasmic 1
IPI:IPI00329801.12	Gene_Symbol = ANXA5 annexin A5
IPI:IPI00465248.5	Gene_Symbol = ENO1 isoform alpha-enolase of Alpha-enolase
IPI:IPI00333541.6	Gene_Symbol = FLNA filamin-A
IPI:IPI00019502.3	Gene_Symbol = MYH9 myosin-9
IPI:IPI00479186.5	Gene_Symbol = PKM2 isoform M2 of pyruvate kinase isozymes M1/M2
IPI:IPI00217966.7	Gene_Symbol = LDHA isoform 1 of L-lactate dehydrogenase A chain
IPI:IPI00257508.4	Gene_Symbol = DPYSL2 dihydropyrimidinase-related protein 2
IPI:IPI00334775.6	Gene_Symbol = HSP90AB1 85 kDa protein
IPI:IPI00219018.7	Gene_Symbol = GAPDH glyceraldehyde-3-phosphate dehydrogenase
IPI:IPI00847435.1	Gene_Symbol = EEF1A1 EEF1A1 protein
IPI:IPI00413958.3	Gene_Symbol = FLNC isoform 2 of filamin-C
IPI:IPI00013808.1	Gene_Symbol = ACTN4 alpha-actinin-4
IPI:IPI00855785.1	Gene_Symbol = FN1 isoform 15 of fibronectin precursor
IPI:IPI00827679.1	Gene_Symbol = VIM 50 kDa protein
IPI:IPI00328415.11	Gene_Symbol = CYB5R3 Isoform 1 of NADH-cytochrome b5 reductase 3
IPI:IPI00221226.7	Gene_Symbol = ANXA6 annexin A6
IPI:IPI00003362.2	Gene_Symbol = HSPA5 HSPA5 protein
IPI:IPI00645078.1	Gene_Symbol = UBE1 ubiquitin-activating enzyme E1
IPI:IPI00844159.2	Gene_Symbol = PGM1 62 kDa protein
IPI:IPI00796333.1	Gene_Symbol = ALDOA 45 kDa protein
IPI:IPI00291006.1	Gene_Symbol = MDH2 malate dehydrogenase, mitochondrial precursor
IPI:IPI00019988.1	Gene_Symbol = SGSH *N*-sulphoglucosamine sulphohydrolase precursor
IPI:IPI00020599.1	Gene_Symbol = CALR calreticulin precursor
IPI:IPI00798430.1	Gene_Symbol = TF transferrin variant (Fragment)
IPI:IPI00477536.2	Gene_Symbol = FLNB isoform 7 of filamin-B
IPI:IPI00554538.3	Gene_Symbol = TPP1 60 kDa protein
IPI:IPI00010796.1	Gene_Symbol = P4HB protein disulfide-isomerase precursor
IPI:IPI00455315.4	Gene_Symbol = ANXA2 annexin A2
IPI:IPI00009342.1	Gene_Symbol = IQGAP1 Ras GTPase-activating-like protein IQGAP1
IPI:IPI00744692.1	Gene_Symbol = TALDO1 transaldolase
IPI:IPI00294578.1	Gene_Symbol = TGM2 isoform 1 of protein-glutamine gamma-glutamyltransferase 2
IPI:IPI00100980.9	Gene_Symbol = EHD2 EH domain-containing protein 2
IPI:IPI00186711.3	Gene_Symbol = PLEC1 plectin 1 isoform 6
IPI:IPI00027442.4	Gene_Symbol = AARS alanyl-tRNA synthetase, cytoplasmic
IPI:IPI00306576.1	Gene_Symbol = ARSB arylsulfatase B precursor
IPI:IPI00291005.8	Gene_Symbol = MDH1 malate dehydrogenase, cytoplasmic
IPI:IPI00383680.3	Gene_Symbol = RPN2 Ribophorin II
IPI:IPI00219077.4	Gene_Symbol = LTA4H isoform 1 of leukotriene A-4 hydrolase
IPI:IPI00395646.1	Gene_Symbol = TXNDC5;MUTED thioredoxin domain-containing 5 isoform 2

Table 1 (*Continued*)

Accession	Name
IPI:IPI00295851.4	Gene_Symbol = COPB1 coatomer subunit beta
IPI:IPI00657860.1	Gene_Symbol = BST1 uncharacterised protein BST1
IPI:IPI00219365.3	Gene_Symbol = MSN Moesin
IPI:IPI00743646.2	Gene_Symbol = TXNRD1 CDNA FLJ46672 fis, clone TRACH3009008, highly similar to thioredoxin reductase
IPI:IPI00023006.1	Gene_Symbol = ACTC1 Actin, alpha cardiac muscle 1
IPI:IPI00455383.4	Gene_Symbol = CLTC Isoform 2 of Clathrin heavy chain 1
IPI:IPI00296099.6	Gene_Symbol = THBS1 thrombospondin-1 precursor
IPI:IPI00001639.2	Gene_Symbol = KPNB1 importin subunit beta-1
IPI:IPI00382470.3	Gene_Symbol = HSP90AA1 heat shock protein 90 kDa alpha (cytosolic), class A member 1 isoform 1
IPI:IPI00218236.6	Gene_Symbol = PPP1CB Serine/threonine-protein phosphatase PP1-beta catalytic subunit
IPI:IPI00827795.1	Gene_Symbol = CD44 isoform 8 of CD44 antigen precursor
IPI:IPI00477531.2	Gene_Symbol = DYNC1H1 532 kDa protein
IPI:IPI00413654.3	Gene_Symbol = SFRS5 Isoform SRP40-4 of Splicing factor, arginine/serine-rich 5
IPI:IPI00784273.1	Gene_Symbol = TLN1 Talin-1
IPI:IPI00644989.2	Gene_Symbol = PDIA6 isoform 1 of protein disulfide-isomerase A6 precursor
IPI:IPI00759754.1	Gene_Symbol = TTN isoform 1 of Titin
IPI:IPI00456429.3	Gene_Symbol = UBA52 ubiquitin and ribosomal protein L40 precursor
IPI:IPI00026216.4	Gene_Symbol = NPEPPS puromycin-sensitive aminopeptidase
IPI:IPI00012102.1	Gene_Symbol = GNS *N*-acetylglucosamine-6-sulfatase precursor
IPI:IPI00219217.3	Gene_Symbol = LDHB L-lactate dehydrogenase B chain
IPI:IPI00749398.1	Gene_Symbol = SERPINB6 serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6
IPI:IPI00848342.1	Gene_Symbol = LTF lactotransferrin precursor
IPI:IPI00644127.1	Gene_Symbol = IARS isoleucyl-tRNA synthetase, cytoplasmic
IPI:IPI00176903.2	Gene_Symbol = PTRF isoform 1 of polymerase I and transcript release factor
IPI:IPI00456695.1	Gene_Symbol = PSMD1 isoform 2 of 26S proteasome non-ATPase regulatory subunit 1
IPI:IPI00848316.1	Gene_Symbol = Glucose phosphate isomerase
IPI:IPI00788802.1	Gene_Symbol = TKT transketolase variant (Fragment)
IPI:IPI00414320.1	Gene_Symbol = ANXA11 annexin A11
IPI:IPI00002557.1	Gene_Symbol = COPG2 coatomer subunit gamma-2
IPI:IPI00471966.3	Gene_Symbol = ACTR3B 47 kDa protein
IPI:IPI00847250.1	Gene_Symbol = FASN Fatty acid synthase
IPI:IPI00007702.1	Gene_Symbol = HSPA2 heat shock-related 70 kDa protein 2
IPI:IPI00848312.1	Gene_Symbol = PLS3 plastin-3
IPI:IPI00784628.1	Gene_Symbol = Myeloid/lymphoid or mixed-lineage leukemia/clathrin assembly protein fusion protein (Fragment)
IPI:IPI00012268.3	Gene_Symbol = PSMD2 26S proteasome non-ATPase regulatory subunit 2
IPI:IPI00867501.1	Gene_Symbol = TUBA1B similar to tubulin alpha-ubiquitous chain
IPI:IPI00298971.1	Gene_Symbol = VTN vitronectin precursor
IPI:IPI00009111.1	Gene_Symbol = TPBG Trophoblast glycoprotein precursor
IPI:IPI00797646.2	Gene_Symbol = GLB1 galactosidase, beta 1 isoform b
IPI:IPI00796463.2	Gene_Symbol = DOPEY2 isoform 2 of protein dopey-2
IPI:IPI00220749.1	Gene_Symbol = ITGA7 isoform alpha-7X1B of integrin alpha-7 precursor
IPI:IPI00383500.1	Gene_Symbol = PLEKHC1 isoform 2 of pleckstrin homology domain-containing family C member 1
IPI:IPI00002520.1	Gene_Symbol = SHMT2 serine hydroxymethyltransferase, mitochondrial precursor
IPI:IPI00853468.1	Gene_Symbol = ARSA arylsulfatase A isoform b
IPI:IPI00003411.3	Gene_Symbol = PTGIS prostacyclin synthase
IPI:IPI00792207.1	Gene_Symbol = ALDH2 mitochondrial aldehyde dehydrogenase 2 variant (Fragment)
IPI:IPI00784008.1	Gene_Symbol = IPO7 importin-7
IPI:IPI00853455.1	Gene_Symbol = CTSD protein
IPI:IPI00169285.5	Gene_Symbol = P76 LAMA-like protein 2 precursor
IPI:IPI00004503.5	Gene_Symbol = LAMP1 lysosomal-associated membrane protein 1
IPI:IPI00029863.4	Gene_Symbol = SERPINF2 alpha-2-antiplasmin precursor
IPI:IPI00217766.3	Gene_Symbol = SCARB2 lysosome membrane protein 2
IPI:IPI00411639.1	Gene_Symbol = RPSAP15 laminin receptor-like protein LAMRL5
IPI:IPI00010157.1	Gene_Symbol = MAT2A S-adenosylmethionine synthetase isoform type-2
IPI:IPI00791490.1	Gene_Symbol = PDXK 29 kDa protein

of Sevag's solution, consisting of 3 parts of *n*-butanol and 1 part of chloroform, to 3 volumes of FlowTS-6. This is a classical method to precipitate proteins so as to purify polysaccharide from natural products [11], but was herein adopted to reversely purify proteins from carbohydrates. After being incubated at 4 °C for 1 h with occasional shaking, the FlowTS-6/Sevag mixture was separated into three phases. The upper aqueous layer was discarded and the mid phase containing proteins was carefully collected and mixed with 3 volumes of pre-cold acetone at −20 °C for 20 min. Then, visible precipitate was harvested by centrifugation (12,000 rpm for 10 min) and air-dried for subsequent use.

2.3. Mass spectroscopy and protein identification

After being quantified by Bradford's assay [12] and examined with SDS-PAGE, the acquired proteins were further analysed with mass spectroscopy (MS) [13]. Briefly, dried peptides were reconstituted in 0.1% formic acid and analysed in a Q-Star Elite mass spectrometer (Applied Biosystems; MDS-Sciex), coupled to the online Tempo™ Nano-MDLC system (Applied Biosystems). Sample separation was conducted on a nanobored C18 column (0.3 μL/min; 120-min gradient) and mass spectrometer was set into the positive-ion mode (mass range: 300–2000 *m/z*; selected charge: +2 to +4). The three most abundantly charged peptides above a 5 count threshold were chosen for MS/MS and dynamically excluded for 30 s with ±30 mmu mass tolerance. Protein identification was performed with ProteinPilot software v2.0.1 (Applied Biosystems) by searching the raw data against the International Protein Index (IPI) human database (IPI_human version 3.34.fasta, including 69,164 sequences and 29,064,824 residues), which also allows for the possibilities of more than 80 biological modifications using the BLOSUM 62 matrix. Only peptides

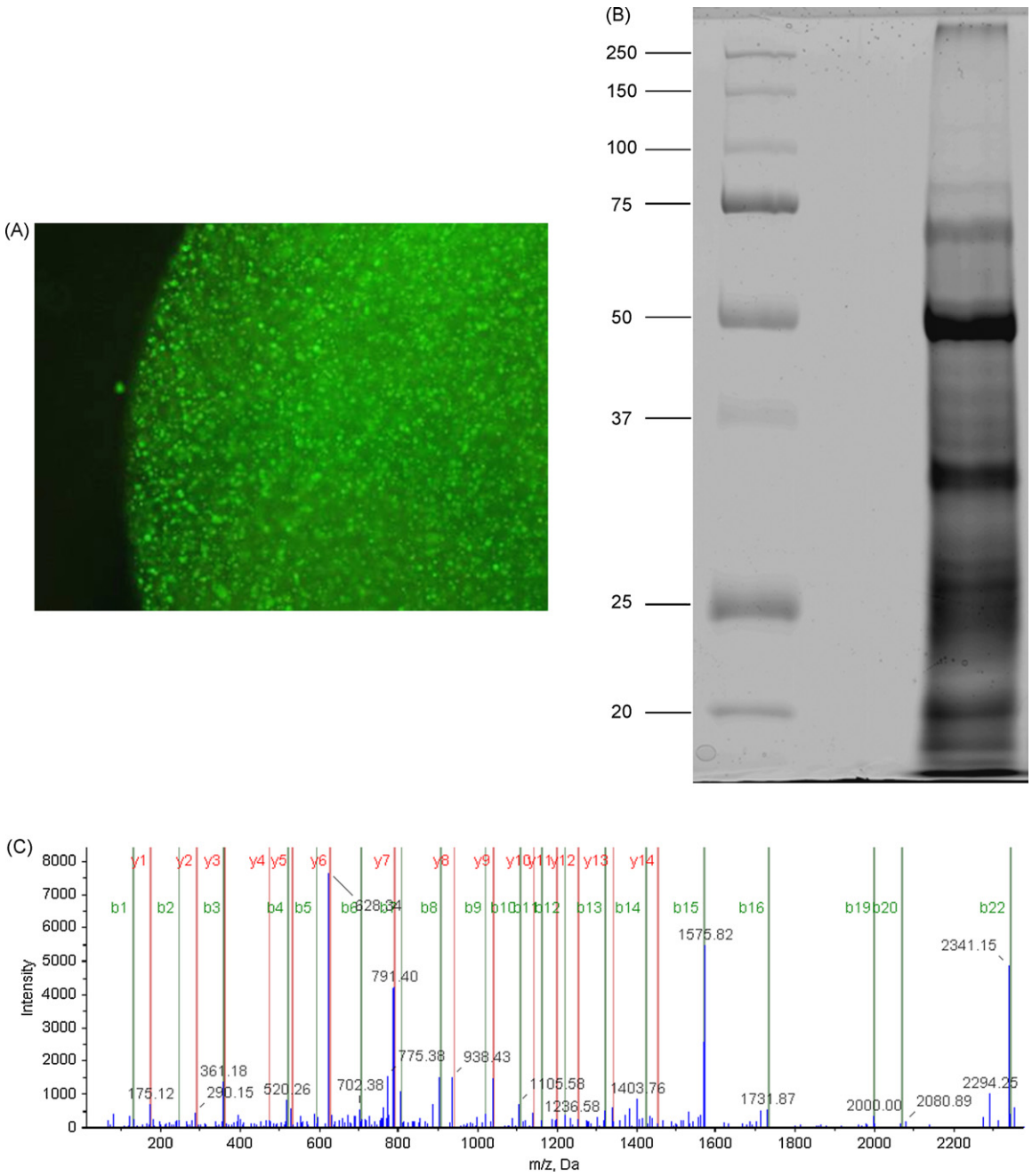

Fig. 2. (A) Live/Dead indication of cell viability; (B) SDS-PAGE gel (10%) was stained with Brilliant Blue G solution [0.1% (w/v) Blue G, 25% (v/v) methanol, and 5% (v/v) acetic acid in deionised water], and sufficiently washed for visualisation; molecular weight markers are from 20 to 250 kDa; (C) mass-spectrum of the fragment of human actin (cytoplasmic 1: KDLYANTVLSGGTTMYPGIADR).

with at least 70% confidence were taken for protein identification.

3. Results and discussion

The results show that proteins were successfully isolated from gel constructs, in which the cells were shown to be highly viable at Day 3 post-encapsulation (Fig. 2A). Bradford's protein quantification indicated that the yield is 1.73 ± 0.11 mg/ml, which is comparable with some previous studies [8]. The bands between 30 kDa and 75 kDa in PAGE gel were intercepted and illustrated in Fig. 2B (Left lane: standard markers; Right lane: the sample; GE-Amersham Imaging System). Outcomes from MS analysis further confirmed that the sample is composed of human proteins, with the spectrum of the actin fragment being selectively presented in Fig. 2C. From this peptide fragment, in total 95 proteins were identified and listed in Table 1. It suggests that our assay is capable of providing proteins of sufficient quality that could fulfil the high requirement of MS, which provides sensitive and informative analysis of proteins and has become an indispensable tool in the current age of proteomics [14]. This is also the reason we chose MS among various techniques of detection. However, the products are also expected to be applied to other subsequent experiments such as 2D-electrophoresis, Western blotting or immunoprecipitation according to the researchers' demands and convenience.

In the above, we straightforwardly reported a novel assay without using any control method because there was no existing, ready-to-use protocol for protein isolation from polysaccharide-

based hydrogel constructs. Some feasible techniques devised for similar condition (e.g., plant tissues) were reportedly useful in protein extraction experiments, but did not result in effective RNA co-extraction [7,8]. Moreover, several challenges were confronted during our preliminary trials, which would be worthwhile being discussed for technical references. First, it is not ideal to skip certain treatment steps and directly precipitate proteins with acetone from FlowTS-6: this may produce white precipitates that are hardly dissolvable (even with incubation at 56 °C for 30 min). One possible explanation could be that the carbohydrate chains, initially invisible, were precipitated by acetone and enlaced the proteins. Second, when a high yield is desired, it is not recommended to pool the flow-through from Step 7 (FlowTS-7) together with that from FlowTS-6, which may introduce more DNA contaminant. As indicated in the manual, Qiagen-Step 7 could be skipped if researchers are to conduct DNA digestion, suggesting that DNA is removed with FlowTS-7. To verify this, we recorded a UV-absorption spectrum of FlowTS-7 and observed a sharp peak at 260 nm, indicating the presence of DNA therein. Proteins were still precipitated and quantified from FlowTS-7, but their content was found to be much lower, ~15% of that from FlowTS-6. Therefore, proteins are mostly contained in FlowTS-6, and those in FlowTS-7 could be neglected and discarded. Third, some researchers, especially phytochemists, prefer the use of trichloroacetic acid (TCA) for protein precipitation. This reagent is not suitable hereto, because the normally used lysis buffers such as TRIzol® or Qiagen-RLT contain guanidine thiocyanate or similar compounds that react with TCA.

4. Conclusion

In summary, we have designed a protocol, based on the reverse application of Sevag's method, to obtain proteins from carbohydrate-rich constructs of engineered tissue. It starts with the discard solution during an established procedure of RNA extraction, and generates protein samples that are of sufficient quality for proteomic analysis. Therefore, in addition to the RNA verification reported in our previous study, we can envisage that this technique allows the concurrent acquisition of both proteins and RNA from carbohydrate-rich engineered tissue. This approach may benefit biomaterialists and tissue engineers in various fields.

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