

Evaluation of egg white ovomucin-based porous scaffold as an implantable biomaterial for tissue engineering

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Abstract: Studies have shown the technological and functional properties of ovomucin (OVN) in the food-agricultural industry. But research has yet to explore its potential as an implantable biomaterial for tissue engineering and regenerative medicine. In this study we isolated OVN from egg white by isoelectric precipitation and fabricated scaffolds with tunable porosity by utilizing its foaming property. Gelatin a known biocompatible material was introduced to stabilize the foams, wherein different ratios of OVN and gelatin had a significant effect on the degree of porosity, pore size and stability of the formed hydrogels. The porous scaffolds were crosslinked with EDC resulting in stable scaffolds with prolonged degradation. Improved cell proliferation and adhesion of rat bone marrow-derived mesenchymal stem cells were observed for OVN containing scaffolds. Although, scaffolds with 75% OVN showed decrease in cell proliferation for L929 fibroblast type of cells. Further biocompatibility assessment as implant material was determined by subcutaneous

implantation in rats of selected scaffold. H&E staining showed reasonable vascularization over time and little evidence of severe fibrosis at the implant site. Persistent polarization of classically activated macrophage was not observed, potentially reducing inflammatory response, and showed increased expression of alternatively activated macrophage cells that is favorable for tissue repair. Analysis of IgE levels in rat serum after implantation indicated minimal and resolvable allergic response to the OVN implants. The results demonstrate OVN as an acceptable implant scaffold that could provide new opportunities as an alternative natural biocompatible and functional biomaterial in various biomedical applications. © 2016 Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater* 00B: 000–000, 2016.

Key Words: ovomucin, biomaterial, hydrogel, tissue engineering

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INTRODUCTION

Ovomucin (OVN) has remained the least studied protein of the egg albumin due to the absence of an easy to scale-up isolation protocol. OVN was typically isolated by ultracentrifugation, gel filtration chromatography, and density gradient electrophoresis.^{1–3} In addition, it is insoluble at neutral pH or in the absence of denaturing agents, limiting its use as a food ingredient.⁴ Recent advances in its isolation and purification protocols has prompted researchers to discover the different biological activities and properties of OVN. At present, OVN is most often isolated from egg white by isoelectric precipitation (pH 5.0–6.0), followed by a simple purification method that involves washing with sodium chloride salt solution, to remove co-precipitating proteins like ovalbumin and lysozyme.^{5,6} It can also be solubilized by

enzymatic hydrolysis and sonication in a mild alkaline solution or in a reducing agent such as mercaptoethanol.⁷

OVN is a macromolecule that comprises 2–4% of the total egg white protein.⁶ It is a sulphated glycoprotein that is subdivided into the carbohydrate-poor α_1 -OVN (MW: 135–150 kDa) and α_2 -OVN (MW: 230.9 kDa), and the carbohydrate-rich β -OVN (MW: 523–720 kDa).^{1,5,8} The sulfated glycopeptides in OVN were found to activate cultured macrophage-like cells⁹ and enhance the proliferation of mouse spleen lymphocytes stimulated by lipopolysaccharide.¹⁰ It has gained high attention as a source of bioactive ingredients, and studies have exhibited cholesterol lowering effects,¹¹ antitumor,^{12,13} antiviral,¹⁴ and antibacterial¹⁵ properties of OVN. Furthermore, this highly glycosylated OVN presents a vast potential for application, as it was

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considered to be one of the main contributors to the gel-like properties of egg whites^{16,17} due to its protein-based foaming and stabilizing properties.¹⁸

The bioactive properties and functions of OVN that have been discovered so far and may yet be uncovered demonstrate high potential for use in tissue engineering, in particular, as a new biomaterial source or hydrogel scaffold. But the suitability of hydrogels as biomedical materials and their performance in a particular application depend to a large extent on their bulk properties.¹⁹ To our knowledge no study exist with the use of OVN as a porous scaffold and has investigated its bulk properties as an implantable biomaterial for tissue engineering. Thus, the first objective of the study was to fabricate stable OVN-based porous scaffolds by using its foaming property to produce the desired pore size and porosity. Gelatin (GEL) was used as copolymer for the system in order to create a stable structure and to improve protein foaming ability, as GEL structure is more susceptible to molecular interactions compared to other proteins with more complex structure like its parent collagen. The foaming properties of proteins are basically associated to their properties of forming films in the air-water interface, and proteins that can be quickly opened and adsorbed present better foaming properties than slowly adsorbed proteins having structures that are more difficult to open.²⁰ In addition, GEL is one of the most widely used biodegradable natural polymers that is typically combined with other polymers to improve mechanical properties.^{21,22} The second objective of the study is to determine the physico-chemical properties and characterize cellular response *in vitro* of the generated OVN and OVN-GEL porous scaffolds. Finally, to assess basic biocompatibility as a potential implantable biomaterial by subcutaneous implantation in a rat animal model and determination of its immune response toward OVN. We hypothesized that these OVN-based scaffolds would be degradable with resolvable inflammation levels due to its open porosity, which promote cell infiltration and better integration with the surrounding tissue. But like any novel material being developed as an implant material, there is a demand for further biocompatibility characterization of the OVN protein *in vivo*, since there have been very few literature in terms of its significance and contribution especially to cases of egg allergy.²³

MATERIALS AND METHODS

Materials

Fresh eggs (70–80 g each) were used for OVN preparation. Sodium chloride (NaCl), 2-mercaptoethanol and sodium phosphate dibasic anhydrous (Na₂HPO₄) were purchased from Daejung Chemicals and Metals, Korea. GEL (from porcine skin, Type A, pI 7.0–9.0), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution, fluorescein isothiocyanate (FITC) conjugated phalloidin, and Hoechst 33342 were purchased from Sigma-Aldrich, USA. Ethanol, acetone, and dimethylsulfoxide (DMSO, 99%) were purchased from Samchun Pure Chemical, Korea. The water used in the entire experiment was deionized through a Milli-Q System (Millipore, USA). All the

other chemicals used were of reagent grade. The primary antibodies used in this study were goat polyclonal CD206 (1:50, Santa Cruz Biotech, Santa Cruz, CA), rabbit monoclonal CCR7 (1:100, Epitomics, Burlingame, CA) and mouse anti-rat CD68 (1:50, Serotec, Raleigh NC). The secondary antibodies used were Alexa Fluor® goat anti-rabbit 568 (Invitrogen), Alexa Fluor® donkey anti-goat 488 (Invitrogen) and Alexa Fluor® donkey anti-mouse 350 (Invitrogen).

Isolation of OVN

OVN (90 ± 2.5% purity) was prepared using the 2-step isoelectric precipitation method previously published^{6,17} under mild conditions and without using toxic chemicals. Briefly, egg whites were separated from the yolk, and stirred with 100 mM NaCl solution for 30 min with the pH adjusted to 6.0. The dispersion was kept at 4°C overnight and separated by centrifugation at 15,300 g for 10 min at 4°C. The precipitate was re-suspended this time in 500 mM NaCl solution and the process was repeated for purification with the final isolated OVN lyophilized and then stored at –20°C until time of use.

Preparation of OVN hydrogels

Stock solution of 5% OVN (w/v) was prepared by mixing lyophilized OVN powder in deionized water with 0.1% of 2-mercaptoethanol solution then sonicated for 10 min. Stock solution of 5% GEL (w/v) was also prepared by dissolving GEL powder in deionized water solution at 37°C. Different proportions of OVN and GEL were mixed, starting with no OVN (OVN-0), 25% OVN (OVN-25), 50% OVN (OVN-50), and 75% OVN (OVN-75). The mixtures were foamed by using a vortex mixer (3200 rpm) for 10 min at room temperature.

Hydrogels were fabricated by immediately pouring the foamed solutions into cylindrical PVC molds (Figure 1) with 15 mm in diameter and 10 mm in height to have a uniform and manageable form. The hydrogels were then frozen at –20°C for 1 h and lyophilized (Ilshin Lab, Korea) for 24 h. The resulting scaffolds were crosslinked by suspending them in 20 mM EDC with 50 mM NHS in acetone-water (4:1). Crosslinking was carried out at 4°C for 24 h. The crosslinking solution was removed and the scaffolds were washed with 0.1 M Na₂HPO₄·12H₂O and 2 M NaCl for 1 h with moderate shaking using a mechanical shaker. The scaffolds were isolated, washed with deionized water for 20 min, and then dehydrated using 50% acetone and neat acetone prior to freeze drying for 24 h.

Characterization of OVN-GEL scaffolds

Samples were cut into cylinders measuring 15 mm in diameter × 5 mm in height, which were used for the succeeding experiments unless stated otherwise. Field emission scanning electron microscope (SEM, JSM701F, JEOL, Japan) was used to evaluate the morphology of the hydrogel scaffolds. Samples were coated using a platinum sputter coater (Cresington Scientific Instruments, UK) prior to observations. All micrographs were taken at an acceleration voltage of 10 kV.

Mercury intrusion porosity meter (Quantachrome Porosity Meter, USA) was used to determine the percent porosity and pore size distribution frequency of the samples, calculated using the accompanying Poremaster software.

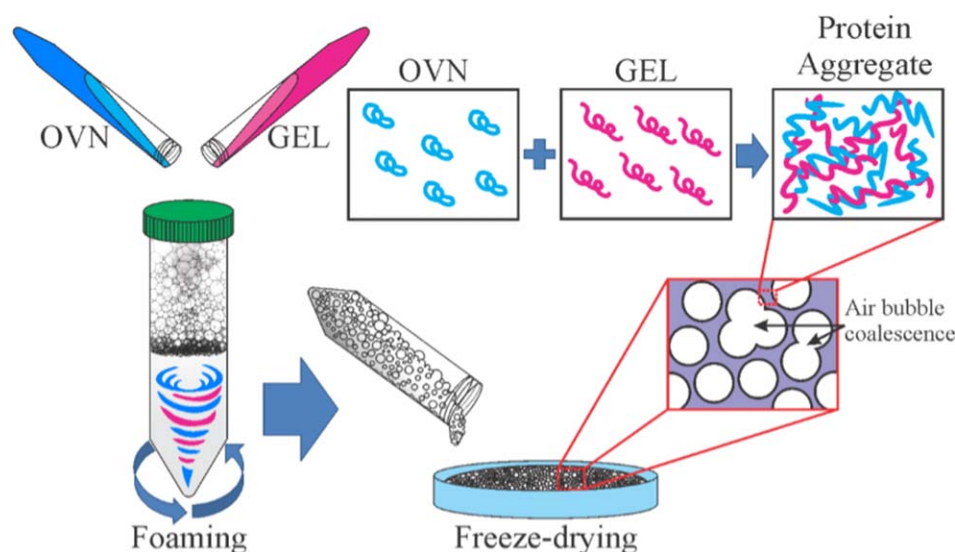


FIGURE 1. Illustration of the scaffold preparation and formation of OVN-GEL protein-based foam.

FTIR (Nicolet iS10-Smart iTR, Thermo Fisher Scientific, USA) spectra were taken for the structure and functional group analysis of the OVN and OVN-GEL samples. Spectra were obtained in the $400\text{--}4000\text{ cm}^{-1}$ region.

The swelling behavior was analyzed by immersing the samples in phosphate buffer saline (PBS, pH 7.0) solution at 37°C . The dimensional change in each of the sample was measured every 15 min using a caliper until no further dimensional change was recorded (90 min). The initial dimensions prior to soaking were subtracted from the measurements after immersion and the relative size increase (%) was reported.

Biodegradation of the samples was determined by immersing the hydrogels in simulated body fluid (SBF) solution (pH 7.0) incubated at 37°C with initial wet weight (W_0), simulating degradation condition by hydrolysis. The weight (W_x) of each immersed sample was monitored every week, for 4 weeks. The relative wet sample weight (%) at each time point was calculated using the equation: $\text{Sample weight (\%)} = W_x/W_0 \times 100$.

Protein uptake was examined by measuring the total amount of proteins associated with the scaffolds. The samples were extensively washed with PBS and allowed to swell in serum-free MEM medium for 24 h in a humidified incubator at 37°C with 5% CO_2 to take expansion into account when calculating the bulk protein concentration. The samples were placed in a 24-well plate with 2 mL MEM medium supplemented with 10% fetal bovine serum and placed in a humidified incubator. After 24 h of incubation, the samples were transferred to a clean 24-well plate and gently rinsed with PBS to remove loosely attached proteins. Proteins in the samples were measured by PierceTM BCA protein assay kit (Thermo S Scientific, USA).

Determination of cell adhesion and proliferation behavior

Bone marrow-derived mesenchymal stem cells (BMSC) culture preparation. BMSC aspirates were isolated from the bone shafts of rat femurs (Samtako Bio, Korea)

following protocols published elsewhere.^{24,25} The cells were isolated from the aspirates by using a cell strainer filter (100 μm nylon, Falcon, NJ), pooled, and centrifuged for 5 min at 1500 rpm. The cell pellet was re-suspended in α -MEM with 10% FBS and 1% PS. The cells were counted by suspending a small aliquot of cells in 5% acetic acid to remove the red blood cells, and mononuclear cells were counted by trypan blue exclusion. BMSC with a cell count of 1.0×10^7 cells were seeded in a culture dish (150 mm) and were cultured for 10–14 days as a monolayer in a humidified incubator (ASTEC, Japan) at 37°C in 5% CO_2 atmosphere. Third passage cells were used in subsequent experiments.

BMSC culture with samples. The effect on proliferation and cytotoxicity of OVN on BMSC was analyzed using MTT assay reagent. BMSC with a density of 1×10^4 cells/mL was seeded on the scaffolds and incubated for 1, 3, and 7 days in 24-well culture plates. Culture media was replaced every other day. For every sampling day, MTT solution was added to the designated samples and re-incubated for 4 h. DMSO was then added on the plates and incubated with agitation for 1.5 h. The absorbance was then read at 595 nm using an ELISA plate reader (Infinite F50, Tecan, Austria).

Cell adhesion behavior was observed through confocal laser scanning microscope (CLSM, FV10i, Olympus, Japan). Samples were seeded with BMSC with a density of 2×10^4 cells/mL and were incubated for 1, 3, and 7 days in 24-well culture plates. For each of the sampling dates, the scaffolds were harvested and fixed with 4% paraformaldehyde in PBS. The cells were permeabilized using 0.5% Triton X-100 and were blocked for non-specific binding with an antibody diluent. The cells were then stained for filamentous actin with FITC, and for the nucleic stain with Hoechst 3342. Samples were mounted and observed using a confocal laser scanning microscope. Low and high magnification

micrographs were taken and analyzed using the accompanying FV10-ASW 3.0 Viewer software.

Evaluation of in vivo response to OVN-GEL scaffolds

Implantation procedure. All procedures were performed in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals and with the approval of the animal care committee at the Soonchunhyang University. Twelve male 8 week-old *Sprague Dawley* rats weighing approximately 250 g (Samtako Bio, Korea) were randomly assigned per scaffold type and incubation period. Each animal was conditioned for 1 week in suitable rat cages supplied with adequate food and water. Rats were anesthetized through ethyl ether inhalation after which the dorsal area of the animal was shaved, cleaned with 70% ethanol and disinfected with povidone-iodine solution. An incision was created along the dorsolateral aspect and a subcutaneous pocket was created through the blunt dissection of the dermal tissue from the underlying muscle layer. Samples measuring 15 mm diameter by 5 mm height were then inserted in the subcutaneous pockets with at least four samples implanted per animal. The incisions were then closed with nylon suture and the animal was allowed to recover. Animals were sacrificed at 14 and 28 days post-surgery. The implant site including the surrounding native abdominal wall tissue was excised and fixed in 10% formaldehyde solution. The sections were then processed for routine paraffin sectioning.

Histological analysis and immunolabelling. Paraffin embedded sections were deparaffinized with xylene and rehydrated through a graded ethanol series. Tissue sections were stained with hematoxylin and eosin (H & E) stain for histological analysis. Micrographs were taken using an Olympus light microscope (BX53) equipped with a digital camera (DP72 Olympus) and analyzed with ImageJ software.

The degree of degradation *in vivo* was estimated by measuring the area occupied by the remaining sample from the stained images. Calculations were made given the dimensions 10 mm diameter by 5 mm height cylinder and the theoretical cross-sectional area of a sample cut along the sagittal plane that is around 50 mm².

Immunofluorescence staining was done to further characterize the macrophage cells surrounding the implanted samples. Heat-mediated antigen retrieval was performed using 0.1 mM EDTA buffer at 95–100°C for 25 min on the corresponding tissue section slides. Tris-Buffered Saline Tween-20 (TBST) washing was done for 15 min, followed by incubation in blocking buffer (1% bovine serum albumin) for 1 h. The primary antibodies (CD68- pan- macrophage marker, CCR7- M1 marker and CD206- M2 marker) diluted in blocking buffer were added to the slides for 16 h at 4°C in a humidified chamber. The slides were then washed three times in PBS prior to the addition of the corresponding secondary antibodies (Alexa Fluor 350, 568 and 488) for 1 h in a humidified chamber at room temperature. The slides were again washed three times in PBS prior to viewing. Six confocal micrographs per observation period

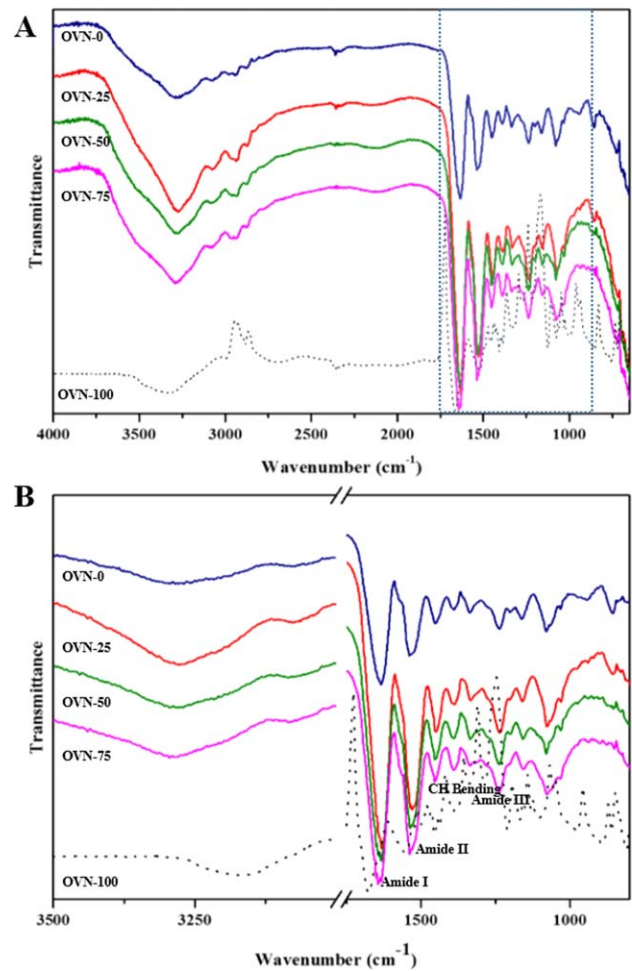


FIGURE 2. (A) FT-IR spectra of different OVN-GEL scaffolds crosslinked with EDC-NHS and (B) highlighted finger print region for crosslinked OVN-GEL.

was taken using FluoView FV10i Confocal Laser Scanning Microscope. Micrographs were analyzed using ImageJ software through a uniform macros for determining the amount of cells expressing both the pan macrophage (blue) and either M1 (red) or M2 (green) stain. Cells expressing both M1 and M2 staining were excluded in the analysis.

Evaluation of serum immunoglobulin levels via ELISA

Sera from scaffold implanted rats were collected at each time point and the IgE levels were quantitatively measured using enzyme-linked immunosorbent assay (IgE Rat ELISA kit, Koma biotech Inc., Korea). Samples and standards were added to each 96-well flat bottom, high binding plates. The pooled reference sera (without implant) were assigned an arbitrary unit value that was used as a standard for all assays performed. All samples were diluted in PBS, incubated for 1 h at room temperature and analyzed in triplicate. Absorbance was read at 405 nm after color development. Sample relative units were calculated based on the linear regime of the arbitrary units generated by the standard curve ($R^2 > 0.9$) from pooled sera of *Sprague Dawley* rats without implant run against rat reference serum.

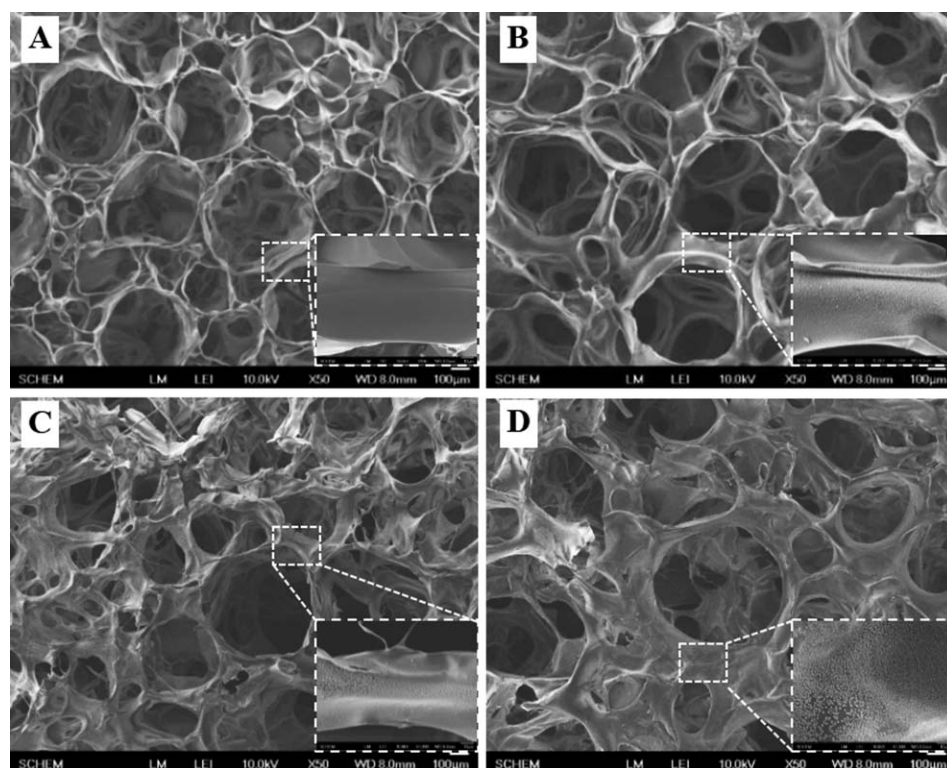


FIGURE 3. SEM micrographs of the OVN-GEL scaffolds (A) OVN-0, (B) OVN-25, (C) OVN-50, and (D) OVN-75 with corresponding high magnifications.

Statistical Analysis

All tests were done using four replicates unless stated otherwise. Statistics were performed using GraphPad Prism 5 (GraphPad Software, Inc., USA). Groups were compared using independent Student's *t* test, with *p*-values <0.05 considered statistically significant. Analysis of variance was used to determine statistical significance of the different scaffolds using M1/M2 ratio.

RESULTS

OVN-GEL scaffold characterization

We used OVN to create porous hydrogels by foaming it with GEL. The different OVN-GEL blends were subjected to freezing at low temperature (-20°C) and lyophilization to preserve foam integrity. The resulting foam hydrogels with weak physical interactions degrade rapidly at physiologic conditions, thus further crosslinking with EDC-NHS was done. FTIR results shown in Figure 2, confirm that crosslinking predominantly occurred in the GEL component as no new peaks were observed at the spectral finger print region for the OVN-GEL hydrogels. Cross linked gelatin (OVN-0) and OVN-100 served as the basis for the observations. The most prominent peaks in this area of the spectrum are around 1634 , 1536 , and 1231 cm^{-1} , which represents the amide groups, Amides I, II, and III, respectively, as well as the CH bending at 1456 cm^{-1} .

The porous structure of the OVN-GEL scaffolds were observed on the SEM micrographs (Figure 3). The scaffolds were highly porous with the formation of thicker textured

trusses as the OVN content increased. The effect of OVN content on the generated pores and porosity were confirmed with the pore size distribution histograms and porosity data [Figure 4(A–E)]. The scaffolds with the highest concentration of OVN (OVN-75) showed micropores with an average diameter of $20\text{ }\mu\text{m}$, as well as macropores of $100\text{--}200\text{ }\mu\text{m}$ in diameter (D). OVN-50 generated micropores with an average of $10\text{ }\mu\text{m}$ in diameter, and $100\text{--}500\text{ }\mu\text{m}$ sized macropores (C), OVN-25 with higher GEL content generated macropores with $50\text{--}100\text{ }\mu\text{m}$ in diameter (B), and a majority of $20\text{--}50\text{ }\mu\text{m}$ pore size for the pure gelatin hydrogel (A). The corresponding porosities of the scaffolds were also measured (E), and OVN-75 has the highest porosity of $58 \pm 5.6\%$, followed by OVN-50 with $45 \pm 6.3\%$, and no significant porosity difference was observed for OVN-25 and OVN-0, with $22 \pm 5.7\%$ and $21 \pm 2.2\%$ respectively.

Hydrogel swelling is a function of network structure, degree of crosslinking, as well as hydrophilicity. The swelling and degradation behavior *in vitro* for each scaffold was observed. The swelling ratio for the hydrogels increased linearly by almost a single fold for the first 45 min [Figure 5(A)] up to 90 min wherein maximum swelling or no significant dimensional change was noted. OVN-50 showed the highest increase in diameter of up to $10.6 \pm 0.62\%$ while the other three samples had no significant difference from gelatin (OVN-0), with an average increase of $7.08 \pm 0.77\%$ in diameter. Likewise, the hydrogels degraded by almost a single fold every week. OVN-75 degraded least after 4 weeks with $60.2 \pm 3.04\%$ remaining sample, $51.2 \pm 2.45\%$

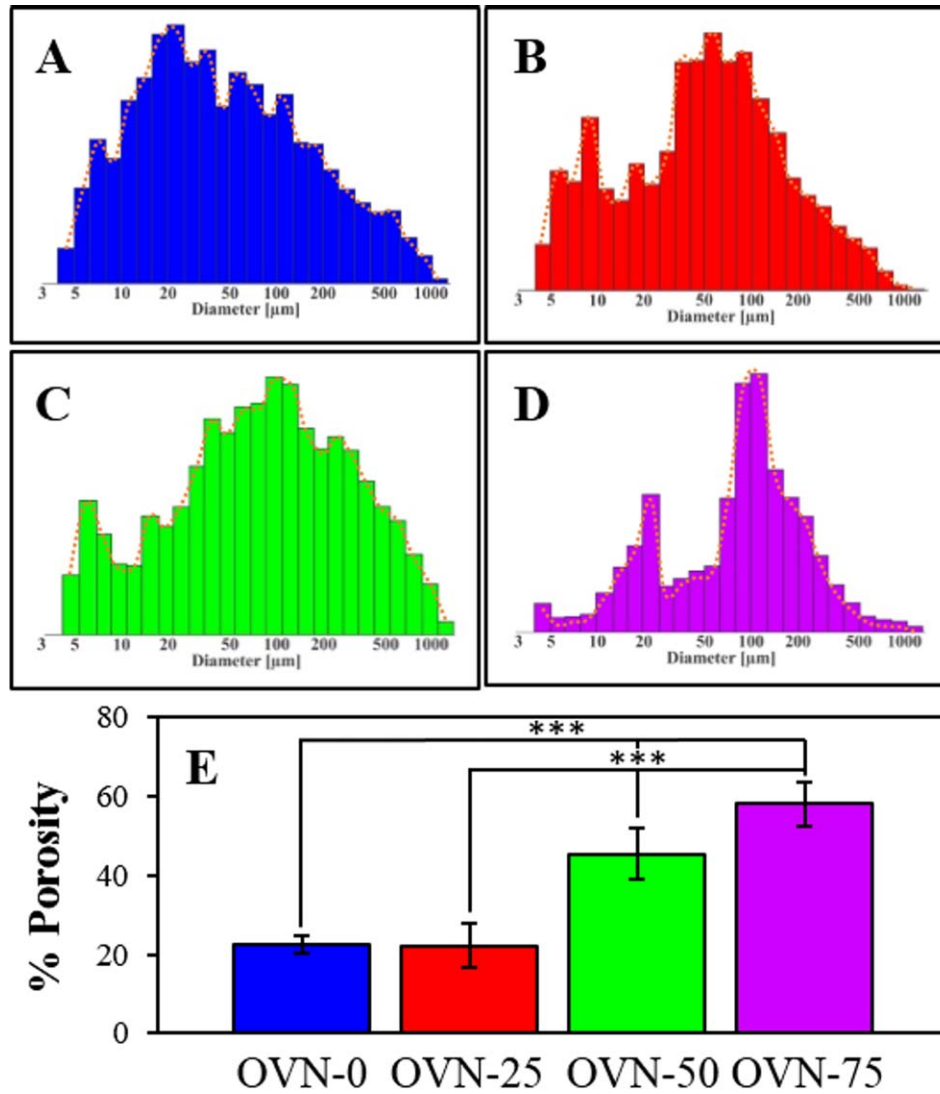


FIGURE 4. Pore size distribution frequency histogram of (A) OVN-0, (B) OVN-25, (C) OVN-50, (D) OVN-75 and their corresponding (E) overall porosity. The values plotted are average $\pm$ SD of four replicates with *** $p < 0.001$.

for OVN-50, $46.6 \pm 4.85\%$ for OVN-25, and $33.6 \pm 3.14\%$ for OVN-0 [Figure 5(B)]. From the data, we can observe that the degradation rate is inversely proportional with the OVN content. On the other hand, the degree of swelling seems to be affected by some other factor.

The amount of adsorbed proteins on the surface and absorbed protein in bulk of the OVN-GEL scaffolds are shown in Figure 5(C). The protein uptake on the surface of the samples were not significantly different, thus the bulk protein uptake was also calculated based on the volume of the samples, to take into account the differences in the network structure. Samples with OVN showed higher protein absorption compared to GEL only ($25.9 \pm 4.07\%$), which can be attributed to the higher porosity of OVN-GEL hydrogels. OVN-75 was with the highest amount of bulk protein uptake ($34.7 \pm 3.29\%$), while bulk protein uptake for OVN-25 ($31.6 \pm 2.63\%$) and OVN-50 ($30.6 \pm 2.20\%$) was not significantly different.

Cell interaction with OVN-GEL scaffold

BMSC were seeded on the samples and analyzed using standard MTT assay to assess cytotoxicity. Based on the results, the scaffolds allowed cell adhesion and proliferation after 1 day of seeding. MTT assay (Figure 6) of the cells on the scaffolds showed no significant difference in proliferation between the various fabricated scaffolds after a day of culture. However, the succeeding sampling days showed an improved proliferation of the cells on all the fabricated samples, with OVN-75 showing the best results.

Increased cell density and further colonization of the cells on the scaffolds were apparent from the first up to the seventh day of culture. Confocal images showed that BMSC have more defined and organized cytoskeletons (well-defined contractile actin microfilaments, and prominent focal contacts) on the scaffolds with higher OVN content: OVN-50 and OVN-75 (Figure 7). The high magnification images showed defined actin fibers and round nuclei of the

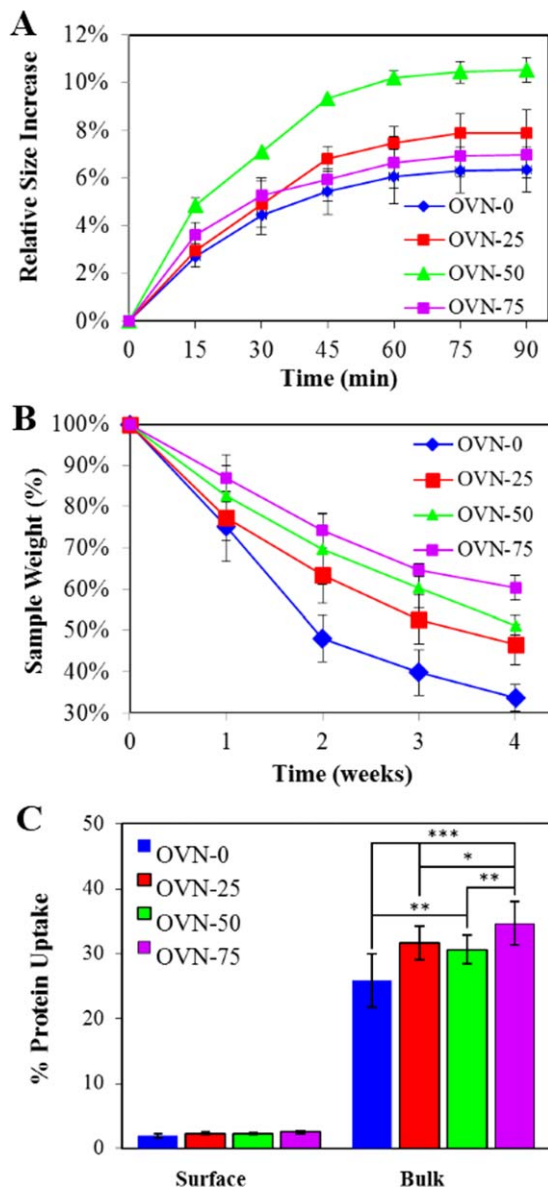


FIGURE 5. (A) Swelling kinetics and (B) *in vitro* degradation by hydrolysis of the different OVN-GEL scaffolds. (C) Quantification of adsorbed protein on the surface and absorbed protein in bulk of the different OVN-GEL scaffolds. The values plotted are average $\pm$ SD of four replicates, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

cells, which indicate cell migration and active cell division. The cell morphology also showed a correlation with surface structure, wherein more cells proliferated on denser surfaces.

In vivo response toward OVN-GEL scaffold

Upon extraction of the subcutaneous implants, most of the GEL samples (control) were almost totally degraded after 2 weeks. Implanted OVN-50 (selected) sample still remained after 4 weeks post implantation. Tissue sections of extracted tissue samples reveal the extent of hydrogel degradation under *in vivo* conditions [Figure 8(D,G)]. Based on the average calculations, the cross-sectional area of the implanted OVN-GEL scaffold was reduced down to $25.2 \pm 8.98\%$ of the

original size two weeks after implantation. Further degradation was observed after 4 weeks in which the cross-sectional area of the remaining samples was calculated to be only $2.90 \pm 0.26\%$.

Examination of the central region and interface of the histological sections of the 2-week OVN-50 samples show minimal cell infiltration with corresponding fibrous tissue formation [Figure 8(F)]. Four weeks after implantation, the sample area and the fibrous tissue appears to have mostly receded and remained localized around the remaining implant [Figure 8(I)]. The implanted OVN-50 samples did not result in the formation of thick persistent fibrosis and the size of fibrous tissue surrounding the sample appeared to be relative to the remaining implanted sample, aside from the presence of macrophage cells. Formation of blood vessels was also observed along the fibrous tissue formed at the periphery and within the central regions after 4 weeks (indicated by arrows).

Immunofluorescence staining of tissue sections [Figure 9(A)] revealed the presence of a large number of macrophage cells surrounding the implanted samples with their respective polarization. Confocal micrographs show the presence of both macrophage cells (blue) that are either classically activated (M1 red) or alternatively activated (M2 green). Based on the results of the image analysis of the micrographs [Figure 9(B)], a $44,944 \mu\text{m}^2$ confocal micrograph contained around an average of 30 macrophage cells in both 2 and 4 weeks extracted samples. Macrophage cells analyzed from the 2 weeks samples showed that $33 \pm 0.09\%$ was found to be classically activated (M1 pro-inflammatory) and $44 \pm 0.10\%$ was alternatively activated (M2-repair). Macrophage cells analyzed from the 4 weeks samples showed that $18 \pm 0.10\%$ was found to be classically activated (M1 pro-inflammatory) and $57 \pm 0.11\%$ was alternatively activated (M2-repair). Analysis of the micrographs indicates a reduction of M1 polarization and alternatively a promotion of M2 macrophage polarization 4 weeks

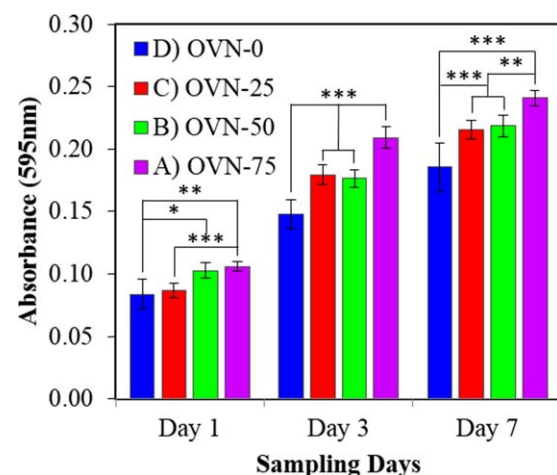


FIGURE 6. Cell proliferation of BMSC cells on the different OVN-GEL scaffolds over a week of incubation tested using MTT assay. The values plotted are average $\pm$ SD of four replicates, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

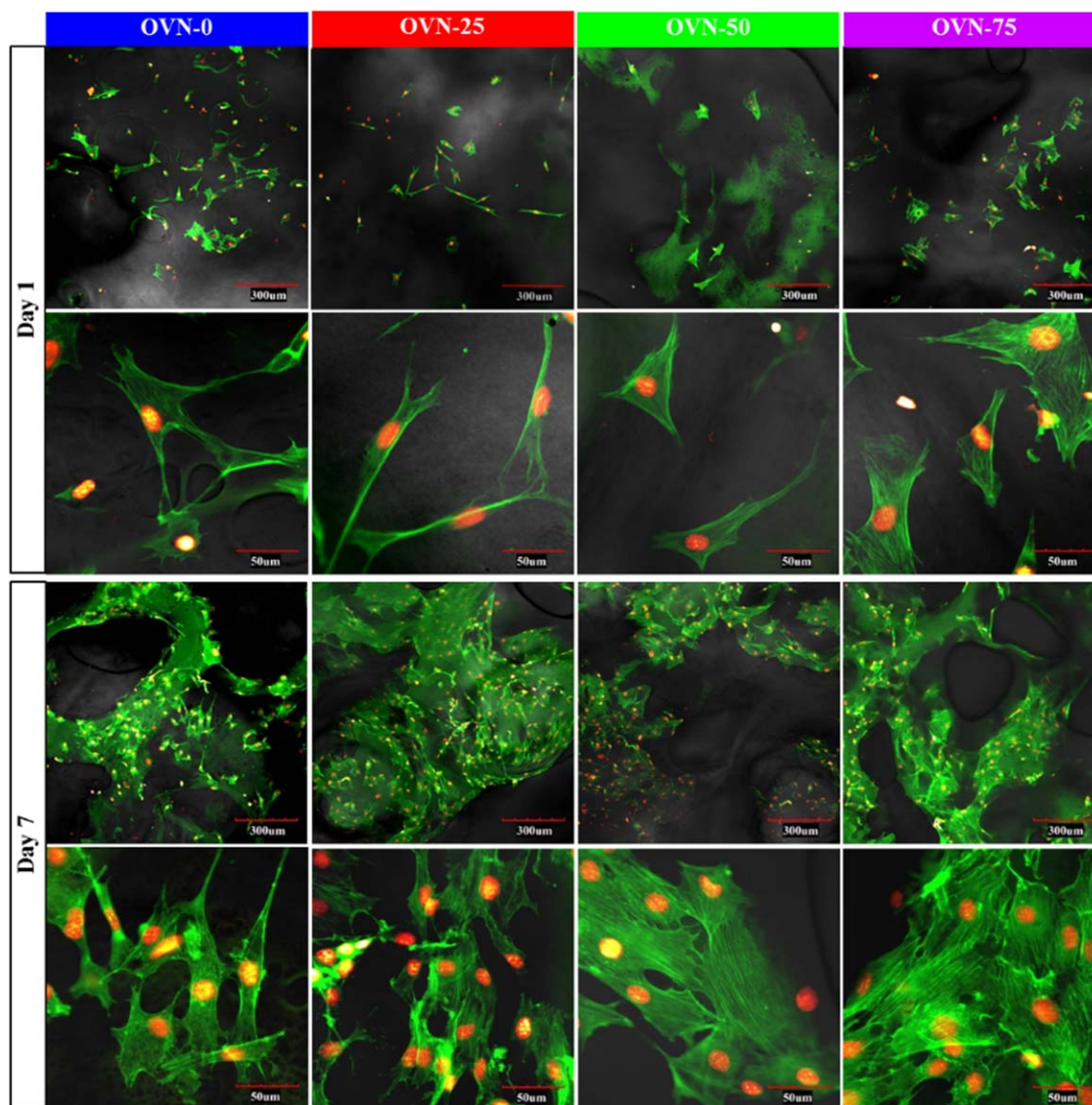


FIGURE 7. Low and high magnification confocal laser scanning micrographs showing morphology of BMSC adhering on OVN-0, OVN-25, OVN-50, and OVN-75 scaffolds after 1 and 7 days of culture.

after implantation. The immunoglobulin assay (IgE), result (316 ± 3.56 kU/L) indicated minimal allergic reaction or response to OVN-50, which corresponds to the remaining scaffold after 4 weeks of implantation, in comparison with the baseline reading for non implanted *Sprague Dawley* rats (196 ± 1.85 kU/L).

DISCUSSION

Continuous research is made on natural based biomaterials, since the clinical performance of most synthetic biomaterials is confined to their ability to support cell adhesion and release growth factors. However, these growth factors are relatively unstable and expensive to be used in their purified and biomaterial-encapsulated form in clinical treatments, and with carcinogenic potential as relatively high doses are needed for therapeutic efficacy.²⁶ In contrast,

natural polymers bear chemical features that have evolved for the purpose of dynamically controlling biological processes that promote new tissue formation.²⁷ Herein, we considered a natural component from egg white, OVN, with functional properties such as emulsifying and gelling,¹⁷ aside from its common use in food systems. Investigations also about the use of OVN as a potential source of biomaterial, have highlighted that it exerts some bioactivity.^{11,15} To our knowledge, the use of OVN's foaming ability to fabricate implantable porous scaffolds have not been explored. The fabrication method by foaming different ratios of OVN and GEL allows for the creation of porous scaffolds with specific pore size and degree of porosity, which is ideal as a tissue engineering platform that requires material and structural properties specific to their particular application.

Protein-based foams consist of air bubbles with each droplet containing a thin and continuous film of protein

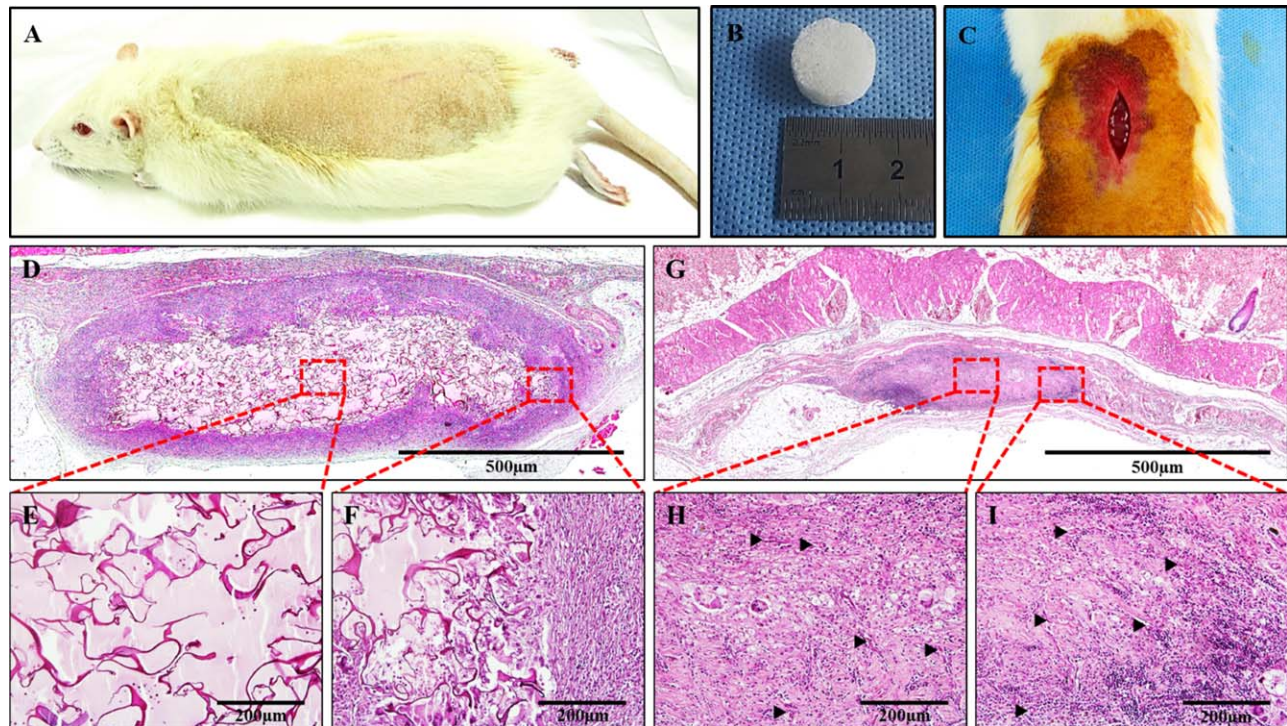


FIGURE 8. Images of percutaneously implanted OVN-50 on rats (A-C), with low and high magnification images of extracted and stained tissue sections after 2 weeks (D-F) and 4 weeks (G-I). Arrows indicate blood vessel formations.

molecules (Figure 1). During OVN-GEL foaming, a protein film surrounding a gas bubble is generated and the gas bubbles are packed together producing the overall structure.²⁰ The presence of thicker hydrogel struts with increasing OVN content (Figure 3) may be attributed to hindrances in

bubble coalescence or thinning of the liquid film between neighboring bubbles, which is affected by the extension of molecular interactions of proteins, that is, OVN and GEL in the air-water interface. The solution containing polydisperse random coils of GEL molecules and OVN forms aggregates

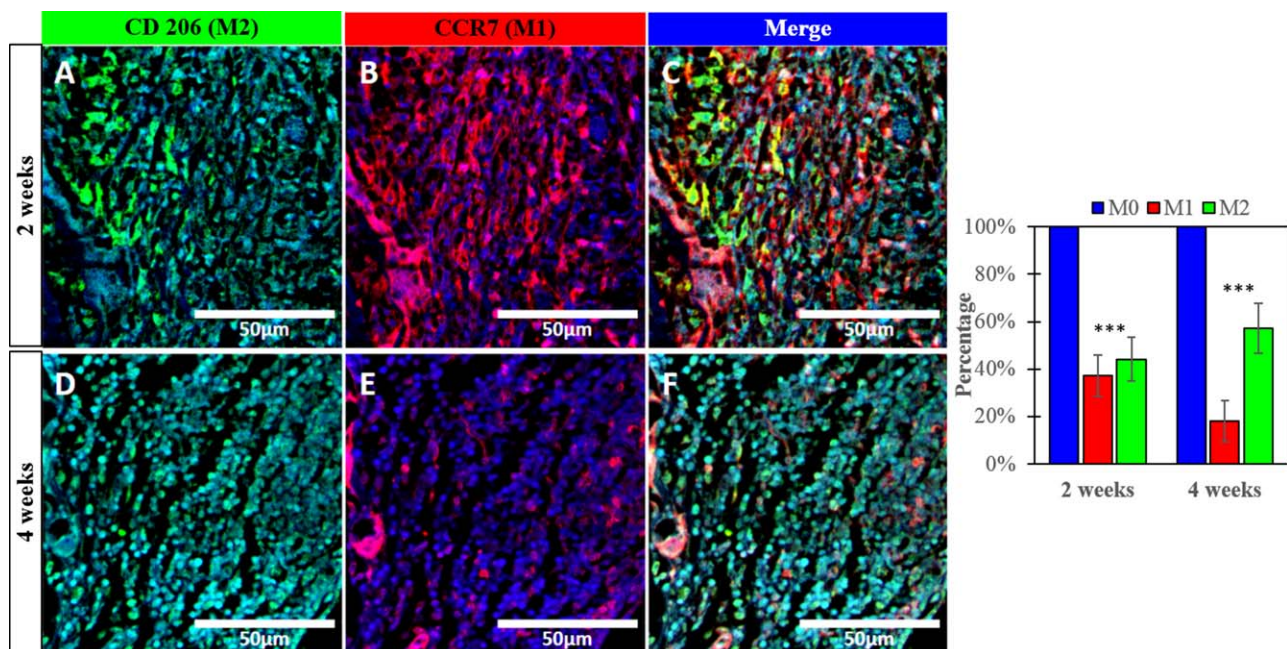


FIGURE 9. (A) Immunofluorescence staining of implanted OVN-50 revealed the presence of large number of macrophage cells surrounding the implanted samples and their respective polarization: pan macrophage, M0 (blue), M1 (red) and M2 (green) stain. (B) Corresponding M0, M1 and M2 quantification based on image analysis of micrographs. The values plotted are average $\pm$ SD of six replicates, *** $p < 0.001$.

of filamentous and fibroid structures leading to foam formation. Furthermore, the coalescence of the protein foams and the velocity of foam formation that is enhanced by OVN, produces larger and more stable pores. This was evidently observed on the pore size distribution frequency (Figure 4), wherein a shift to the right of the pore distribution frequency, with higher pore size range was observed with increasing OVN content.

The suitability of hydrogels as biomedical materials and their performance in a particular application depend to a large extent on their bulk properties. Several studies have shown that the effectiveness of a biomaterial is coupled with the successful invasion of cells, and adequate transport of gases, nutrients and waste products in and out of the biomaterial and eventually the formation of vascularized tissue. These generally rely on the scaffold porosity and pore architecture. Studies suggest an optimum pore size of 5 μm for neovascularization, 5–15 μm for fibroblast ingrowth, 20–125 μm for regeneration of adult mammalian skin, 100–350 μm for regeneration of bone, 40–100 μm for osteoid ingrowth, and 20 μm for hepatocytes ingrowth.^{19,28} The fabricated OVN-GEL scaffolds possess these aforementioned optimum pore size ranges (Figure 4) for successful cell infiltration and growth. Since we are mostly considering the use of OVN as material for either bone or skin tissue engineering, we based evaluation on the general consensus of approximately 60% porosity threshold for sustainable bony integration with resorbable materials, wherein OVN-50 and OVN-75 adhere to. The presence of these types of porosity had already been proven to be beneficial for vascularization and cell adhesion.^{29,30} This coincides with the observed cell proliferation (Figure 6) and adhesion results with BMSC, wherein OVN-50 and OVN-75 were the most promising, with highly organized cytoskeletons (Figure 7). According to a widely accepted observation, the better organized the cytoskeleton of the cells, the faster they could spread and divide.³¹ This was consistent with what we have observed on the OVN-GEL surface, where BMSC exhibited more spreading morphology, and higher cell adhesion and proliferation when compared with only GEL. Furthermore, the results were in unison with previous reports on the ability of OVN to support cell proliferation using mouse spleen lymphocytes.¹⁰ But further tests using differentiated cell type, such as L929 fibroblast cells, reveal relatively low cell proliferation with OVN-75 scaffolds. We can infer from this result that OVN-75 was less suitable for skin tissue engineering applications. In addition, swelling profile of OVN-75 [Figure 5(A)] reveals that at high OVN content, swelling is mainly affected by network composition rather than structure. This could be an advantage in terms of protein absorption, which was evident in the protein uptake results [Figure 5(C)]. But taking note that this is bulk protein, it could still prevent attachment of some specific type of proteins that may be essential for other cell types. This information might prove valuable upon more comprehensive examination and analysis of protein attachment for a target application that is not covered in this study. Thus, we selected OVN-50 for implantation and further biocompatibility

test, so as to firstly determine the behavior *in vivo* of an OVN porous scaffold with a more definitive property, and also limit the number of animals that will be used for the experiment for ethical concerns.

The porous structure of the OVN-GEL scaffolds allowed cells to infiltrate into the matrix and more readily degrade the scaffold. These scaffolds were also well integrated after 4 weeks (Figure 8), with evident vascularization that correlated with the amount of fibrous tissue surrounding the implant. The fibrous tissue was not persistent indicating that the stimulated inflammatory response was only present in the initial stage. The M1/M2 analysis (Figure 9) also supports this observation, wherein M1 macrophage primarily associated with inflammatory response was high at 2 weeks but reduced after 4 weeks. The cytokines and stimuli, M1 and M2, have different roles in the development, maturation, and activation of macrophages. Integration of these isolated stimuli is necessary to represent the complex changes that macrophages undergo during full activation.³² At 4 weeks the M2 response for OVN-50 increased that could be indicative of immunoregulation, allergic response, or matrix deposition and tissue remodeling. No allergic symptoms or tendencies were observed physically on the rats. IgE levels of the pooled rat sera were analyzed to further investigate possible immunoglobulin recognition, and minimal reaction was found that correlates with the remaining implant sample.

The implanted OVN-GEL scaffold show promise as a versatile natural biomaterial implant for tissue engineering applications. The OVN-GEL scaffolds can be easily fabricated by foaming method, as well as further modified via chemical crosslinking. These scaffolds were also well integrated at 4 weeks and appeared biocompatible with resolvable inflammation levels, which can be attributed to the open porous design of the scaffolds, allowing cells to infiltrate into the matrix and more easily degrade the scaffold, and thus support tissue remodeling over time. Further investigation will involve testing OVN-GEL scaffolds in more functional applications to evaluate the degradation kinetics and biological response at other implant sites.

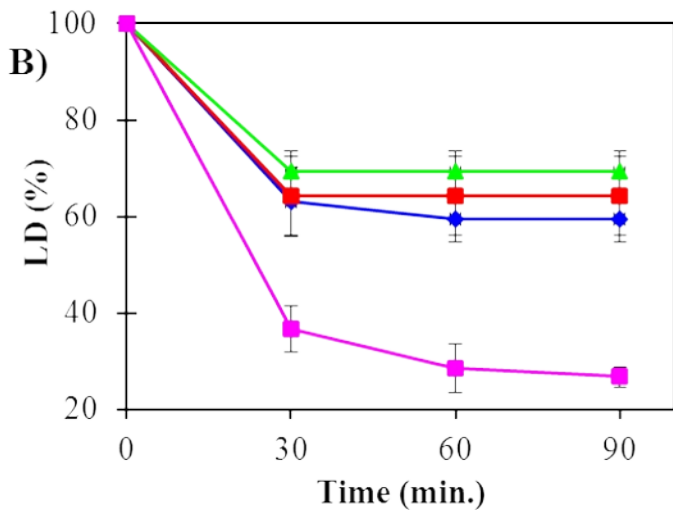
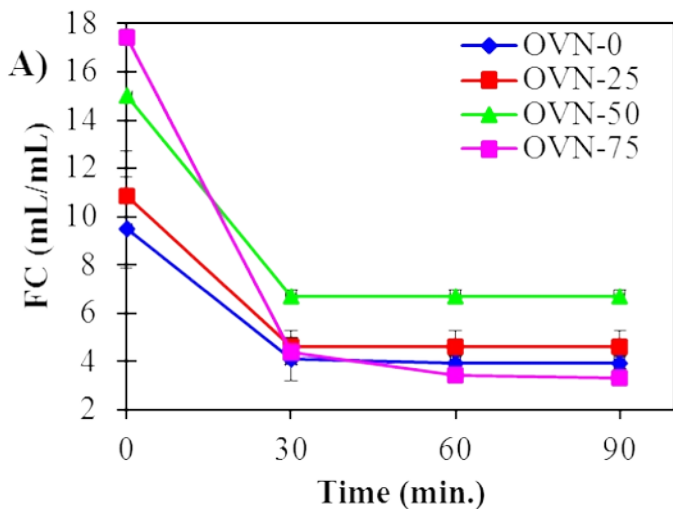
CONCLUSION

The structural properties of OVN-GEL scaffolds can be altered by different OVN-GEL ratios. The ratio of OVN to GEL was an influential factor in the size of the generated pores and the overall porosity of the formed hydrogels, consequently affecting swelling, degradation and biocompatibility. Increasing the amount of OVN that is mixed with GEL generated larger pore size with thicker pore walls and improved the stability of the hydrogels. *In vitro* biodegradation test showed an inverse relationship between the degradation rate and the amount of OVN, while swelling kinetics and protein absorption for higher OVN content was more dependent on the surface and network composition. BMSC proliferation was relatively better with OVN containing scaffolds, indicating good biocompatibility *in vitro*. Scaffolds with equal ratios of OVN to GEL were the more stable and

suitable for the target applications, which was further evaluated *in vivo*. Inflammatory reaction was acceptable and macrophage response toward repair was evident. Thus, we have successfully fabricated a biocompatible porous hydrogels by foaming OVN and GEL that has potential for further development as an implant material for either bone or skin tissue engineering. A more comprehensive look on the foaming property in combination with other proteins for targeted applications will be done in our future studies. Long term *in vivo* study is also underway.

REFERENCES

- Kato A, Ogino K, Matsudomi N, Kobayashi K. Separation of ovomucin into carbohydrate rich and poor components by chromatography on lysozyme-sepharose 4 B. *Agric Biol Chem* 1977;41:1925–1929.
- Eichholz A. The hydrolysis of proteids. *J Physiol* 1898;23:163–177.
- Robinson DS, Monsey JB. The composition and proposed subunit structure of egg-white beta-ovomucin. The isolation of an unreduced soluble ovomucin. *Biochem J* 1975;147:55–62.
- Kato A, Nakamura R, Sato Y. Studies on changes in stored shell eggs part VII. Changes in the physicochemical properties of ovomucin solubilized by treatment with mercaptoethanol during storage. *Agric Biol Chem* 1971;35:351–356.
- Abeyrathne EDNS, Lee HY, Ahn DU. Sequential separation of lysozyme, ovomucin, ovotransferrin, and ovalbumin from egg white. *Poultry Sci* 2014;93:1001–1009.
- Omana DA, Wu J. A new method of separating ovomucin from egg white. *J Agric Food Chem* 2009;57:3596–3603.
- Hiidenhovi J, Hietanen A, Mäkinen J, Huopalahti R, Ryhänen EL. Hydrolysis of ovomucin by different enzymes. Proceedings of the XVII European Symposium on the Quality of Poultry Meat AND XI European Symposium on the Quality of Eggs and Egg Products. Doorwerth, The Netherlands: WPSA; 2005. p. 251–256.
- Omana DA, Wang J, Wu J. Ovomucin—A glycoprotein with promising potential. *Trend Food Sci Technol* 2010;21:455–463.
- Tanizaki H, Tanaka H, Iwata H, Kato A. Activation of macrophages by sulfated glycopeptides in ovomucin, yolk membrane, and chazae in chicken eggs. *Biosci Biotechnol Biochem* 1997;61:1883–1889.
- Otani H, Maenishi K. Effects of hen egg proteins on proliferative responses of mouse spleen lymphocytes. *LWT Food Sci Technol* 1994;27:42–47.
- Nagaoka S, Masaoka M, Zhang Q, Hasegawa M, Watanabe K. Egg ovomucin attenuates hypercholesterolemia in rats and inhibits cholesterol absorption in Caco-2 cells. *Lipids* 2002;37:267–272.
- Watanabe K, Tsuge Y, Shimoyamada M, Ogama N, Ebina T. Antitumor effects of pronase-treated fragments, glycopeptides, from ovomucin in hen egg white in a double grafted tumor system. *J Agric Food Chem* 1998;46:3033–3038.
- Yokota T, Ohishi H, Watanabe K. Antitumor effects of β -subunit from egg white ovomucin on xenografted sarcoma-180 cells in mice. *Food Sci Technol Res* 1999;5:279–283.
- Tsuge Y, Shimoyamada M, Watanabe K. Bindings of ovomucin to newcastle disease virus and anti-ovomucin antibodies and its heat stability based on binding abilities. *J Agric Food Chem* 1997;45:4629–4634.
- Ryoko K, Makoto H, Yukiko K, Kazuo K, Noriyuki I, Tsutomu O, et al. Inactivation composition of food poisoning bacteria. In: Patent J, editor. Japan: Japanese Application Publication; 2004. A61K 38/00 ed.
- Geng F, Huang X, Majumder K, Zhu Z, Cai Z, Ma M. Mass spectrometry and two-dimensional electrophoresis to characterize the glycosylation of hen egg white ovomacroglobulin. *J Agric Food Chem* 2015;63:8209–8215.
- Offengenden M, Wu J. Egg white ovomucin gels: Structured fluids with weak polyelectrolyte properties. *RSC Adv* 2013;3:910–917.
- Shan Y, Ma M, Huang X, Guo Y, Jin G, Jin Y. Simple pH treatment as an effective tool to improve the functional properties of ovomucin. *J Food Sci* 2012;77:C740–C745.
- Annabi N, Nichol JW, Zhong X, Ji C, Koshy S, Khademhosseini A, et al. Controlling the porosity and microarchitecture of hydrogels for tissue engineering. *Tissue Eng Part B, Rev* 2010;16:371–383.
- Alleoni ACC. Albumen protein and functional properties of gelation and foaming. *Scientia Agricola* 2006;63:291–298.
- Zaman HU, Beg MDH. Improvement of physico-mechanical, thermomechanical, thermal and degradation properties of PCL/gelatin biocomposites: Effect of gamma radiation. *Radiat Phys Chem* 2015;109:73–82.
- Zhu J, Marchant RE. Design properties of hydrogel tissue-engineering scaffolds. *Exp Rev Med Device* 2011;8:607–626.
- Walsh BJ, Hill DJ, Macoun P, Cairns D, Howden MEH. Detection of four distinct groups of hen egg allergens binding IgE in the sera of children with egg allergy. *Allerg et Immunopathol* 2005;33:183–191.
- Alhadlaq A, Mao JJ. Mesenchymal stem cells: Isolation and therapeutics. *Stem Cell Dev* 2004;13:436–448.
- Park SH, Sim WY, Park SW, Yang SS, Choi BH, Park SR, et al. An electromagnetic compressive force by cell exciter stimulates chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. *Tissue Eng* 2006;12:3107–3117.
- Lakatos PL, Miheller P. Is there an increased risk of lymphoma and malignancies under anti-TNF therapy in IBD? *Curr Drug Target* 2010;11:179–186.
- Meikle ST, Standen G, Salvage J, De Santis R, Nicolais L, Ambrosio L, et al. Synthesis and characterization of soybean-based hydrogels with an intrinsic activity on cell differentiation. *Tissue Eng Part A* 2012;18:1932–1939.
- Whang K, Healy KE, Elenz DR, Nam EK, Tsai DC, Thomas CH, et al. Engineering bone regeneration with bioabsorbable scaffolds with novel microarchitecture. *Tissue Eng* 1999;5:35–51.
- Bignon A, Chouteau J, Chevalier J, Fantozzi G, Carret JP, Chavassieux P, et al. Effect of micro- and macroporosity of bone substitutes on their mechanical properties and cellular response. *J Mater Sci Mater Med* 2003;14:1089–1097.
- Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. *Biomaterials* 2000;21:2529–2543.
- Sastry SK, Burridge K. Focal adhesions: A nexus for intracellular signaling and cytoskeletal dynamics. *Exp Cell Res* 2000;261:25–36.
- Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Rep* 2014;6:13.



Supplementary Information

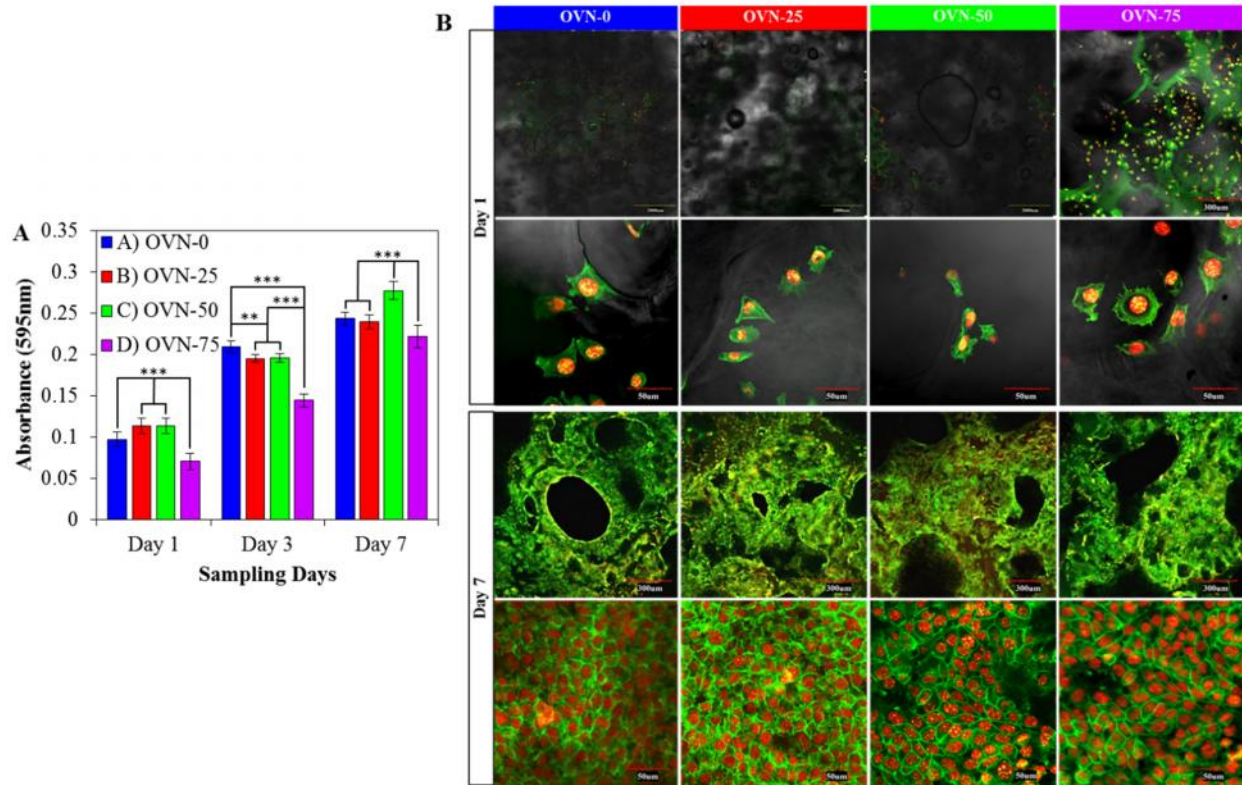


Figure S1. (A) Cell proliferation of L929 cells on the different OVN scaffolds tested using MTT assay. (B) Low and high magnification confocal laser scanning micrographs of L929 fibroblast cells adhering on OVN-0, OVN-25, OVN-50 and OVN-75 scaffolds after 1 and 7 days of culture. The values plotted are average $\pm$ SD of four replicates, ** $p < 0.01$ and *** $p < 0.001$.