Egg white coagulum: a precisely tailorable membrane for biomimetic multilevel structured nanomaterials

Xiaolei Wang, Hui Zhu, Xuexia Liu, Fan Yang & Xiurong Yang

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, 5625 Renmin Street, Changchun, Jilin, 130022, China.

For the first time, hen egg white coagulum was utilized as a surface modification agent for biomimetic multilevel structured nanomaterials (BMSN). By using a straightforward thermal control process, hen egg white can be coagulated in a precisely tailored manner, which is specifically adapted to the morphology of BMSN. Consequently, the structural stability, hydrophobicity and biocompatibility of BMSN can be improved significantly and simultaneously within one hour. Meanwhile, their initial structure-related function can be maintained with higher reliability. These advantages offer an incentive to use egg white coagulum as a facile, precise, quick and much cost-effective alternative to the conventional stabilization materials, such as hot melt adhesive, chitosan and polydopamine.

The research of functional nano-material is heralded as ushering in a “new era,” or even the next industrial revolution. In recent years, substantial efforts have been devoted to the development of biomimetic materials with multilevel structure. These exquisite materials revealed some unexpected properties in a variety of technological and biomedical applications. For instance, by using new developed movable magnetic printing (MMP) technology, biomimetic multilevel structured nanomaterial (BMSN) with “nest-like” morphology was uniformly manufactured in batches. The as-prepared BMSN can provide intelligent protection against pathogenic infection, UV-induced damage, and even electromagnetic irradiation. Unfortunately, as a common problem throughout many exquisite products, the fragility has severely limited the practical applications of this promising material.

Surface modification has been demonstrated as a powerful means to improve the quality of many nanomaterials. However, compared with a large number of surface stabilization studies on zero-dimensional (0D) nanoparticles or one-dimensional (1D) nanostructure, until now, very limited data are available in the area of more complex BMSNs. It remains a great challenge to find a rational agent to stabilize complex structured nanomaterials with minimized structural perturbation. In view of the practical feasibility, this stabilization material should be inexpensive, non-toxic, eco-friendly and can be supplied abundantly from natural sources. Meanwhile, the correlated stabilization procedure is intended to be a mild, quick, facile and straightforward process, which can be conveniently integrated into the manufacture procedure of BMSNs. Aiming at these requirements, for the first time, we proposed a rational BMSN stabilization strategy by using the coagulum of hen egg white (HEW).

In comparison with some commonly used stabilization materials, the applications of HEW in material science or nanotechnology have rarely been explored. However, if we take some experiences from biological engineering and food science, we notice that, some components of HEW are naturally suited as the stabilization material. Apart from water, around 10% weight of the HEW is protein, including ovalbumin, mucoproteins and lysozyme, which is an adhesive and biocompatible liquid medium with antibacterial properties. More importantly, when HEW is intentionally heated, several biochemical reactions occur, involving an initial unfolding of the protein, and the final formation of the gel coagulum. The obtained coagulum can be defined as a thermo-irreversible material containing a continuous and well-defined solid network. In food technology, such network structured coagulum has been widely applied as a versatile binding material. Therefore, it is also anticipated to use HEW as a peculiar heat-triggered material to stabilize BMSNs.

Results

As a proof-of-concept trial, HEW coagulation process was used to stabilize biomimetic “nest-like” nanostructure. The corresponding technological process was described in Figure 1. The coagulation was accomplished...
through a two-step heating process. Magnetic nanoparticles thus could be conveniently removed before solid coagulum formed. The scanning electron microscope (SEM) image of the as-prepared BMSNs was provided in Figure 2a. Nano-arrays with uniform “nest-like” units were formed precisely on the substrate. For each nest structure, well-ordered ZnO nanowires were joined together by the coagulum layer (Figure 2b, red arrow pointed area). On the other hand, the central area of the nest was not sealed by the coagulum, indicating that the HEW layer was also tailored by the movable magnetic nanoparticles. Therefore, Ag decorated SiO$_2$ nanospheres (Figure 2c) can be still exposed at the bottom of the nest (Figure 2b, blue square enclosed area). Consequently, the structure-related bacterium/human cell selective ability can be maintained after HEW coagulation. As a control system, we also prepared similar structured ZnO/Ag/SiO$_2$ BMSN without HEW layer coagulum. In this case, ZnO nanowires, as the fundamental building blocks of BMSN, were simply piled on top of each other (Figure 2d), which was structural vulnerable to external forces.

Figure 1 | The schematic diagram of the MMP process integrated with egg white coagulation. (a) Magnetic particle is attached on the substrate by using magnet force. (b) The growth manner of the subsequent nanostructure would then be defined by the “spacial hindrances” of the magnetic particle. (c) After the formation of magnetic particle printed nanostructure, the surface of the substrate is sprayed with hen egg white. (d) Mild heating is then introduced to start coagulation. (e) The magnetic particle is removed by reversing the magnetic force direction, the coagulum of egg white is then completed with stronger heating. (g) Finally, egg white coagulum stabilized nanostructure with magnetic particle printed morphology will be formed.

Figure 2 | (a) SEM image of nest-like BMSN stabilized by HEW coagulum. (b) SEM enlarged view of a single “nest” unit constructed by ZnO nanowires, which were jointed with each other by means of the surface covered HEW coagulum layer. (c) Detailed view of the Ag decorated SiO$_2$ nanospheres loaded at the bottom of the nest, the image was characterized by SEM coupled with area-scanning composition analysis, while Ag elements were shown by blue color. (d) SEM image of the similar structured BMSN without HEW stabilization.

Figure 3 | (a, b) The optical images of glass substrates covered with bare ZnO/Ag/SiO$_2$ BMSNs (M1) and HEW layer stabilized ZnO/Ag/SiO$_2$ BMSNs (M2) before and after durable tests. The inserts are the contact angle of water on each material surface. (c, d) The SEM images of M1b and M2b. (e) The microbiological toxicity comparison between the M1 and M2 before and after durable test. $V_{E.coli}$, $V_{S.aureus}$ and $V_{HEK293}$ represent the relative cell viability of E. coli, S. aureus and HEK 293 respectively. $S_{HEK293}$ represents the relative average succinic dehydrogenase (SDH) activity (mitochondrial function) of each HEK 293 cell. For ease of comparison, each result on the control (conventional ZnO nanoarrays) was designated as 100% scale. The data are the mean of at least three independent experiments.
To evaluate the differences in durability between BMSNs with or without HEW layer stabilization, glass substrates covered with bare ZnO/Ag/SiO2 BMSNs (M1a) or HEW layer stabilized ZnO/Ag/SiO2 BMSNs (M2a) were immersed in water solution for 5 days at 25 °C and then dried under a flow of nitrogen gas. Serious peeling and fragmentation was observed on M1 (Figure 3b), while a uniform surface was retained and only slight peeling (Figure 3b, green square enclosed area) was observed on the M2 surface. For more reliable quantitative data, similar experiments were repeated for four times in parallel. Based on the gravimetric analysis, over 77.4 ± 2.7% of the BMSNs was lost on the surface of M1. While the loss rate of BMSNs on the surface of M2 was only 3.8 ± 0.6%. Compared with the visual examination, the discrepancies in microstructure between M1b and M2b were even more pronounced. The initial nest-like structure of M1 was completely destroyed (Figure 3c). In contrast, M2b exhibited remarkable structural stability even adjacent to the peeling area (Figure 3d, yellow square enclosed area). Microbiological toxicity experiments on M1 and M2 with and without durable tests were then carried out by Escherichia coli (E. coli), Staphylococcus aureus (S. aureus) and HEK 293. As summarized in Figure 3e, along with the damage of the BMSNs, the relevant bacterium/human cell selective ability is also lost on M1b. In comparison, M2b still exhibits high antibacterial activity against pathogenic bacteria, and low cytotoxicity to human cell. These experiments indicate that HEW coagulation combined with MMP can form a precisely tailored coverage, which is specifically adapted to the complex morphology of BMSN. The structure-related function of BMSN thus can be maintained with considerably higher reliability.

Apart from the structural stability improvement, HEW layer also provided some additional capabilities. One noticeable change is the improvement in surface hydrophobicity (the inserts of Figure 3a and Figure 3b). Surface hydrophobicity is a highly demanded property in practical applications since it is a realistic avenue to enhance the environmental stability, reduce maintenance cost and decrease risks for pollution. To further elucidate these improvements, the two step thermal treatment induced changes in HEW layer were investigated at molecular level. The contents of sulphhydryl (SH) groups, which played an important role during the heat-induced HEW coagulation process, were measured by using 5', 5-dithiobis (2-nitrobenzoic acid) (DTNB) test. Meanwhile, the changes in the secondary structure of HEW were studied by circular dichroism (CD) measurements (Figure 4a). The results were analyzed and summarized in Figure 4b. After the two step heat treatment, the surface SH groups, especially the insoluble fractions, were remarkably increased. In the mean time, a noted increase of β-sheet structure in sacrifice of helical structure was also observed. According to the previous reports, the β-sheet structure of HEW is a well organized network, which is strengthened by heat-exposed hydrophobic residues. Such HEW coagulation thus is capable to improve the structural stability and hydrophobicity of the BMSN simultaneously.

More importantly, we also noticed that, the cell viability of HEK 293 on M2 was considerably higher than that on M1 (Figure 3e). This meaningful discovery can be explained by the main active ingredient of HEW. As the most abundant protein in HEW, ovalbumin is a high-biocompatible scaffold for a variety of biomedical engineering applications. Therefore, it is reasonable to assume that HEW layer can improve the biocompatibility of BMSNs. To test this assumption, pre-osteoblasts were cultured on M1a and M2a surfaces. Bare glass substrate was used as the control system. The relative in-vitro viability tests and morphological observations showed no significant difference between M2a and glass (Figure 5), indicating the good biocompatibility of HEW coagulum. In contrast, considerably fewer attached cells were found on the surface of M1. Collectively, HEW coagulum thus is demonstrated as a versatile membrane that can improve the durability, hydrophobicity and biocompatibility of its covered material simultaneously.

**Discussion**

For the first time, the coagulation of HEW was developed as a mild, eco-friendly and straightforward strategy to improve the durability of BMSNs. By adjusting the heat setting temperature and MMP timing, the layer of HEW coagulum can be formed in a precisely tailored manner, which is specifically adapted to the complex morphology of BMSN. Compared with other commonly used stabilization materials, such as hot melt adhesive, chitosan and polydopamine, HEW is a high biocompatible, abundant and very...
low cost raw material that can improve the structural stability, hydrophobicity and biocompatibility simultaneously. The entire coagulation process can be accomplished within 1 hour through a facile heating-controlled process. These attractive characteristics make HEW coagulum ideally suited for environmental, biomedical and industrial applications. The ongoing research is focused on the extension of HEW coagulation treatment to flexible substrate.

Methods

Characterization. Scanning electron microscopy (SEM) coupled with an energy-dispersive spectroscopy (EDS) analysis were taken using Philips XL 30 and a JEOL JSM-6700F microscope. The microbiological toxicity of the samples was studied by a Leica DMIRE 2 inverted microscope. Optic images were obtained using Panasonic DMC-GF2 camera. The wheatbody were measured by a contact angle meter (CA; Solon information technology Co., Ltd, SI-2800). UV-vis absorption spectra were recorded on a Cary 500 UV-vis NIR spectrometer (Varian, U.S.A.). Circular dichroism (CD) spectra were measured with a 62A DSCD spectrometer (AVIV Company, USA).

The operation of MMP integrated with HEW coagulation. In a typical experiment, the substrate was ultrasonicated consecutively in acetone, ethanol and de-ionized water each for 20 minutes. After dried in the microwave region (500W, 20 seconds × 3 times), Examine methemoglobin/ethanol (50 mM) solution was doped on the substrate surface with AgNO3 (0.05 g/L) covered SiO2 nanoparticles (average diameter = 1.1 μm, purchased from Bausi Company). After 12 hour UV irradiation, the obtained taupe layer was fixed by using hot melt adhesive. The substrate was then covered by layers of proper sized filter (2–15 μm in pore size, purchased from Qinyuan Company). Then the mixture of water, ethanol and magnetic nanoparticles (1–10 μm in diameter, purchased from Baseline Company) was doped on the filter covered substrate. They were fixed by the magnetic force and immersed into the solution with zinc nitrate hydrate (0.025 M) and HMTA (hexamethylenetetramine) (0.025 M) at 80 °C for 12 h. The entire growth and patterning process thus can be accomplished simultaneously. After that, surface of the substrate was removed from solution, immersed in deionized water (5 °C) immediately, and dried at 40 °C overnight. The obtained substrate was then sprayed with liquid egg white (obtained from fresh organic eggs bought locally), with 1.5 M NaCl (pH = 7.5). After that, a mild heating (65 °C 5 min) was introduced to start coagulation. The magnetic particle was then removed through external magnetic fields. The coagulation of egg white was completed with stronger heating (85 °C 30 min).

Cells culture. Bacteria are potentially hazardous and should be treated carefully. Standard bio-safe lab techniques were followed while handling with them and the corresponding media. All microbiological toxicity tests were performed in triplicate to ensure reproducibility. Before the assays, bacterial cultures (E.coli and S. aureus) were grown in solid Luria-Bertani (LB) media overnight at 37 °C with continuous shaking at 200 rpm. The HEK293 cells were maintained in DMEM medium supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and 100 mM non-essential amino acids. The medium was exchanged once every two days. Before the assays, the cells were incubated in a humidified incubator at 37 °C and 5% CO2 atmosphere for 2 days. MC3T3-E1 mouse calvarial preosteoblasts were cultured in 2-minimal essential media (HyClone), containing 10% fetal calf serum (Hyclone) and 1% penicillin/streptomycin (PS) under 37 °C, 5% CO2 environment.

Microbiological toxicity tests. Different BMSN covered substrates were divided into 1cm × 1cm forms and used directly as additives for toxicity test. The HEK 293 cells were first washed three times by using DMEM without serum to get rid of the unattached cells, then 2 mL of cell solution were added into each well the 24-well plates, and the images were taken at certain areas from the wells in order to count the number of cells before incubation, followed by locating different membranes of nanostructures on the bottom of the wells. All the samples were incubated at 37 °C in 5% CO2 the culture. After certain hours of incubation, the cells were removed with Dulbecco’s Phosphate Buffered Saline (DPBS). Then 1 mL DMEM were added into the wells as nutrition medium. The images of cells after incubation were taken from the same area as that before incubation. To evaluate the cytotoxicity of the nanostructures, fluorescent probes (Syto 9/PI) were used to access the live and dead cells. All the samples were studied by a Leica DMIRE 2 inverted microscope. For bacterial cultures, equal densities of S. aureus and E. coli (based on OD600 nm values) were used to inoculate LB broth with different substrates. Broth cultures were incubated with shaking, and viable cell densities were measured via FITC/PI staining. Green fluorescence was detected at 488 nm excitation with a band pass filter ranging between 505 and 530 nm. Simultaneously, red fluorescence was detected using the long pass filter at 585 nm and red superimposition of both green and red fluorescence generated the final images. After treated with different substrates for the same time, the cells in different wells were counted respectively in a haemocytometer, then 5 × 105 cells were transferred to the polypropylene centrifuge tubes for mitochondrial activity assessment. It was measured by using the 3-(4, 5-dimethyl-thiazol-2) -2, 5 diphenyl tetrazolium bromide (MTT) method (Bouillaguet et al., 2000). Each experiment was done in triplicate. The average mitochondrial (SDH) activity per cell was expressed as a percentage of the control cultures.

Studies on thermally induced changes in HEW. The contents of SH groups or protein fraction were measured in duplicate using the methods described by Yoninori Mine (J. Agric. Food Chem. 1997, 45, 2924–2928) and Marc E. G. Hendrickx (J. Agric. Food Chem. 2005, 53, 5726–5733). 1 mL of the 1% protein solution was added with 4 mL of 0.1 M Tris-glycine buffer (pH 8.0) containing EDTA or urea before incubated at 40 °C for 30 min. 125 μL of DTNB (Aldrich) solution (20 mg in 5 ml of 0.1 M Tris-glycine buffer, pH 8.0) was then added and incubated at 25 °C for 10 min. The color absorbance was read at 412 nm to calculate SH contents. CD spectra were measured at protein concentration of 0.5 mg/mL using a cell with a 0.10-cm light path in the wavelength range 205–255 nm. The changes in the different contents of the secondary structure of HEW were interpreted according to the procedure of Chiang et al. (Anal. Biochem.1978, 91, 13–31).

Preosteoblast culture and viability assay. This part of experiments was based on the research works of Adv. Funct. Mater. 2010, 20, 2132–2139 and Adv. Mater. 2003, 15, 1832–1835). MC3T3-E1 mouse calvarial preosteoblasts were seeded at a density of 1 × 104 cells per well and cultured for 2 days. The cell viability was determined using a MTT assay. 50 mL of the MTT solution (5 mg/mL in phosphate buffered saline (PBS)) was added to each well, and the cells were incubated additionally for 3 h at 37 °C. The resulting formazan crystals were dissolved in dimethylsulfoxide, and the absorbance was measured at 595 nm using a Victor 3 microplate reader (Perkin Elmer Inc., Waltham, MA, USA). The morphology of MC3T3-E1 osteoblasts was observed by a confocal laser scanning microscopy (CLSM, Leica DMIRE 2). Before observation, the cell-attached samples were rinsed by sterile phosphate buffered saline (PBS) and stained by fluorescein diacetate (FDA).

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**Author contributions**
X.L.W. and X.R.Y. conceived the idea and designed the project. X.L.W., X.X.L. and H.Z. carried out the experiments. X.L.W., H.Z., X.X.L. and F.Y. analyzed data. X.L.W., F.Y. and X.R.Y. wrote the paper.

**Additional information**
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