Natural Triterpenoid-Tailored Phosphate: In Situ Reduction of Heavy Metals Spontaneously to Generate Electrochemical Hybrid Gels

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Supporting Information

ABSTRACT: In this work, we reported a biocompatible nature product-based soft material which could convert heavy metals to nanoparticles (NPs) in situ spontaneously in a simple step. We have designed and synthesized a natural triterpenoid-tailored phosphate (methyl glycyrrhetate phosphate (MGP)), and this amphiphilic MGP could form the stable hydrogel and extract gold salt from water, followed by spontaneous in situ AuNP formation without external reductants. Notably, the AuNPs were mainly localized on nanofibers instead of gel cavities, and the resulting MGP–AuNPs hybrid gel exhibited attractive electrocatalytic and conductive properties. In addition, as an efficient leaching extraction agent, MGP hydrogel showed higher affinity toward heavy metals over other common metals on account of the high reduction potential of heavy metals. Our work not only provides a novel yet simple way in generating electrochemical hybrid gels by in situ reduction of heavy metals spontaneously but also expands the application of nature product-based functional materials.

KEYWORDS: triterpenoids, gels, nanoparticles, electrochemistry, self-assembly

INTRODUCTION

Low-molecular-weight hydrogels consist of 3D network structures by small molecules to immobilize water and have attracted much attention for their wide potential applications, especially in conductive materials.1−3 Generally, there are three major approaches in fabricating conductive gels, namely, mixing conductive units into a gel, or assembly of electroactive organic components, or in situ synthesis of the conducting system via self-assembly.4−7 In comparison with the former two approaches, the latter one is exceptionally promising because it not only needs less effort in synthesis but also affords highly organized conductive structures on the nanoscale. Among them, metal NPs is one of most widely studied owing to the great potential to conduct and the ability to be constructed in situ.6−12 On the other hand, the compatibility with water renders hydrogels as ideal candidates in environmental remediation for water purification, and a considerable amount of work have been reported where gels were utilized to remove pollutants effectively.13−16 Inspired by above applications in conductive materials and environmental remediation, we wonder if a hydrogel could remove heavy metal ions from water initially, which then were reduced spontaneously to form NPs in situ, and consequently, the resulting hybrid gel has the potential electronic applications.

As a kind of natural products, triterpenoids exist abundantly in licorice root and own great biocompatibility, chiral rigid scaffolds, multiple reactive sites, and unique stacking manners.17−19 The reactive sites (hydroxyl and carboxyl groups) on triterpenoids allow for the easy modification with functional units to advance the noncovalent interactions, thus engendering supramolecular assemblies. In this regard, a number of organogels and hydrogels have been fabricated by triterpenoid over the past 10 years.20−24 For example, pyridinium-appended glycyrrhetinic acid (GA, one kind of triterpenoids) could form the nanofibers in physiological phosphate-buffered saline, and these fibers further entangled with each other to afford the supramolecular hydrogel,22 whereas the GA/β-cyclodextrin host–guest complex directed...
the formation of self-healing supramolecular gels.\textsuperscript{24} However, to date, the fabrication of triterpenoid-based hybrid materials with metal NPs in situ is still rare. As far as we know, only in 2015, Mezzenga and co-workers found that the hydrogel of sugar-containing glycyrrhizic acid was an efficient scaffold for the in situ preparation of hybrid nanomaterials because of the reductive diglucuronic moiety in glycyrrhizic acid.\textsuperscript{23} Thus, developing natural triterpenoid-tailored gels that could reduce heavy metals in situ spontaneously to generate hybrid gels is of great significance.

Herein, in this research, an amphiphilic triterpenoid-tailored phosphate, methyl glycyrrhetate phosphate (\textit{MGP}, Figure 1), was designed and synthesized (synthetic details see \textit{Supporting Information}), where the hydrophilic phosphate moiety was modified on 3-position of hydrophobic methyl glycyrrhetate. The results showed that \textit{MGP} could form the stable hydrogel and remove Au salt from water, followed by spontaneous in situ AuNP formation, nucleated by nanofibers without external reductants. The AuNPs were mainly localized on gel fibers and the resulting hybrid gel exhibited attractive electrocatalytic and conductive properties, which was rare that AuNPs formed in situ spontaneously in a triterpenoid-tailored gel without external reductants. Additionally, as an efficient leaching extraction agent, \textit{MGP} owned higher affinity toward heavy metals over other common metals and displayed no obvious toxicity, which provides a potential way in converting heavy metals to electrochemical hybrid gels in a simple step.

\section*{MATERIALS AND METHODS}

\textbf{Materials.} GA, methyl iodide, phosphorus oxychloride, agarose, chloroauric acid, and other reagents were local commercial products, and they were used as received.

\textbf{Methods.} NMR spectra were recorded on a JEOL JNM-ECA 400 apparatus in dimethyl sulfoxide (DMSO)-\textit{d}_6 at 25 °C. Electrospray ionization mass spectroscopy (ESI-MS) was analyzed on a Bruker ESQUIRE-LC spectrometer in positive mode. High-resolution MS (HRMS) was measured in positive mode using quadrupole time-of-flight as a mass analyzer. Inductively coupled plasma mass spectrometry (ICP-MS) was recorded in Thermo X Series ICP-MS. Fourier transform infrared (FTIR) spectra were measured with a PerkinElmer Spectrum 100. Attenuated total reflection (ATR)-FTIR was recorded on Bruker VERTEX70. UV–vis spectra were measured with a Thermo Scientific Nanodrop 2000C. Gas chromatography–mass spectrometry (GC–MS) was performed on Agilent 6890GC equipped with 5975MSD.

Atomic force microscopy (AFM) was performed on a NanoScope 3D AFM (Veeco, USA) using the tapping mode with a SiN\textsubscript{3} tip (radius of 10–20 nm). Transmission electron microscopy (TEM) images were obtained on a Hitachi H-7600 electron microscope operating at the accelerating voltage of 80 kV. High-resolution TEM images were obtained with a Tecnai G2 microscope at 200 kV.

Rheological experiments were performed in a plate geometry (diameter 25 mm) on the rheometer plate using a TA instrument. Quantum chemistry computations were performed by using B3LYP density functional theory with the Gaussian 09 suite of programs. The structure was optimized by employing the 6-31G (d,p)\textsuperscript{+} basis set in the gas phase.

X-ray diffraction (XRD) was analyzed using a Bruker D8 ADVANCE instrument. The X-ray beam generated with a rotating Cu anode at the wavelength of the KR beam at 1.5418 Å was directed toward the film edge, and the data were analyzed and interpreted by using the Bragg equation.

X-ray photoelectron spectroscopy (XPS) was obtained on VG ESCALAB 250. Wide spectra survey scans were measured at pass energy of 100 eV and spot size 500 μm. The electron flood gun was on to compensate for the charging effect.

Electrodes were prepared as follows: 10 μL of hydrosol of \textit{MGP} in DMSO/water (1:3, v/v) was added dropwise on the surface of carbon paste electrodes (CPEs, diameter 3 mm) and cooled down to room temperature, consequently generating a stable gel. Deionized (DI) water was added on the top to remove DMSO for 24 h, during which the top solution was exchanged for 12 times. After that, an aqueous solution of HAuCl\textsubscript{4} (20 μL, 10 mM) was added dropwise on the modified electrode to interact with the fibers for 16 h for the formation of NPs. Before the measurement, excess heavy metal ions were removed by immersing the modified electrodes into DI water for 3 h.

Cyclic voltammetric measurements were performed in a 50 mL beaker using CHI600E electrochemical workstation. A nonrotatory CPE, AgCl/Ag (saturated KCl), and a platinum wire mesh cage were employed as the working electrode, the reference electrode, and the counter electrode, respectively. The electrolyte solution was 100 mM sodium acetate aqueous solution. Before the measurement, free Au\textsuperscript{3+} ions was removed from gel pockets by immersing the modified electrode in DI water, preventing the electrochemical formation of bulk gold on the electrode. The measurement was scanned at 20 mV/s between −0.40 and 1.0 V for 5 cycles.

Prior to conductance measurements of the nanocomposites, an electrode was fabricated (see \textit{Supporting Information}). The electrode comprises a pair of two blocks each with a dimension of 10.0 cm × 1.0 cm × 1.0 cm, fixed onto a plastic plate (16.0 cm × 10.0 cm) at 10.0 cm apart. The distance between the sides of the two blocks is 1.0 cm. In these experiments, the diameters of all gels were 0.8 cm and the lengths were 1.5 cm. To bridge the graphite blocks, \textit{MGP}–AuNPs gel was placed between them, and a DDS-307 conductivity meter was used to measure the conductance in triplicate.

\textbf{Cell Viability.} Fibroblast cell line 3T3L1 was grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum in 5% CO\textsubscript{2} atmosphere at 37 °C. Cells were seeded with a density of 1 × 10\textsuperscript{4} cells per well in a 96-well culture plate. After overnight incubation, the medium was replaced by fresh DMEM containing \textit{MGP} at certain concentrations and incubated for 24 h. Then, the CellTiter-Blue reagent (20 μL) was added, and the incubation lasted for another 3 h to perform the cell viability assay. The fluorescence intensity was measured at 560/590 nm (\textit{E}_6/E_\text{ref}) using a BioTek Synergy H1 microplate reader.

\textbf{Cell Imaging.} Fibroblast 3T3L1 cells were seeded with a density of 5 × 10\textsuperscript{4} cells per well in a 12-well culture plate. After overnight incubation, DMEM containing 600 μM \textit{MGP} was used to treat cells and then co-cultured for 24 h at 37 °C. After that, the samples were stained with fluorescein diacetate (FDA, 5 μg mL\textsuperscript{-1}) and propidium iodide (PI, 10 μg mL\textsuperscript{-1}) solution. A LSM 700 confocal laser scanning microscope was employed to take images with the excitation of 488 and 555 nm for FDA and PI, respectively.
**RESULTS AND DISCUSSION**

Typically, the gelation ability was investigated as follows: MGP was initially dissolved in pure or mixed solvents, and then the mixture was sonicated for 20 s. After a simple heat–cool process, the mixture was incubated at rt to test whether the gelation occurred, and the results are summarized in Figure S1 and Table S1. The stable gel of MGP formed in DMSO/water, whereas partial gels formed in dimethylformamide (DMF)/water, tetrahydrofuran (THF)/water, methanol/water, and acetone/water under the same concentration (1 wt %, 1:3, v/v). In the following study, the gel in DMSO/water was chosen and its morphologies at different concentrations were investigated by AFM (Figure S2). With the concentration varying from 0.025 to 1 wt %, assemblies of MGP transformed from spheres to nanofibers, and the diameter was around 30 and 15 nm for spheres and fibers, respectively.

For application in reducing heavy metals from water, DMSO was removed from the gel by adding DI water on the top and standing for 48 h, during which the top solution was exchanged for DI water, consequently resulting in MGP hydrogel with around 0.87% (v/v) DMSO determined by GC (Figures S3 and S4). This hydrogel displayed excellent stability and did not exhibit any loss in mechanical integrity according to the rheological results. As can be seen from Figure S5, before and after perfusion with water, the storage modulus $G'$ was always greater than the loss modulus $G''$, which indicated the viscoelastic network of gels. Moreover, the $G'$ for DMSO-removed MGP hydrogel was around 30 times higher than the one before perfusion, suggesting it was a strength-enhanced physical hydrogel. AFM images revealed that fibrils were highly entangled and randomly oriented (Figure 2a,b), and individual fibrils at the edge of the hydrogel were observed clearly, with the width in the range of 15–21 nm and the length at around several micrometers (Figure 2c,f), which were consistent with the ones in TEM and high-resolution TEM images (Figures 2d,e and S6).

FTIR spectra of the xerogel and the powder of MGP were compared to study its driving force. Upon gelation, the saturated C–H stretching vibrations at 2995 and 2910 cm$^{-1}$, assigned to methyl and methylene groups, moved to 2949 and 2872 cm$^{-1}$, respectively, indicating that the van der Waals forces among neighboring MGP backbones were enhanced (Figure S7a). In addition, the stretching vibration of P–O was weakened and shifted by 13 cm$^{-1}$ (1310 to 1323), as well as a 40 cm$^{-1}$ shift for OH (3460 to 3420), revealing the existence of hydrogen bonding originated from phosphates. It could be further confirmed by temperature-dependent $^3$P NMR spectra, where the chemical shift of P gradually moved upfield with the temperature increase (Figure S7b), owing to the collapse of hydrogen bonding. To get deeper understanding, XRD was performed. As shown in Figure S7c, Bragg reflection peak revealed the lamellar structure in the hydrogel network with a $d$-spacing of 3.62 nm. On the basis of the assumption that MGP molecules adopted a fully extended conformation with the molecular length 1.79 nm (optimized by Gaussian 09, Figure S7d), the length of MGP dimer without overlap would be 3.58 nm, which was close to $d$-spacing 3.62 nm. Such a result suggested that MGP molecules assembled into nanofibers by the basic bilayer structures with the phosphate groups on the outside surface.

After having the character of MGP hydrogel in hand, its ability to sequestrate Au$^{3+}$ ion was tested by measuring the uptake from chloroauric acid solution on top of the hydrogel under ambient conditions for 2 days (HAuCl$_4$, 10 mM, 1.0 mL; MGP 1 wt %). The uptake was monitored by analyzing its supernatant using ICP-MS, and its maximum uptake capacity was around 88 μg mg$^{-1}$ (metal: gelator). As shown in Figure 1, the gel color changed to crimson as Au$^{3+}$ diffused into it, the characteristic of AuNPs.$^{29,30}$ To explore the role of nanofibers in the formation of AuNPs in situ, the redox reactions of MGP with HAuCl$_4$ were performed in different solvents; the results of which are summarized in Table S2. No AuNPs appeared when MGP was completely dissolved in DMSO, DMF, THF, methanol, or acetone; while violet stable gels and partial gels were formed in mixed solvents (DMSO/water, DMF/water, THF/water, methanol/water, and acetone/water, 1:3, v/v), revealing the formation of AuNPs. Because fibrous structures only existed in these mixed solvents as seen from AFM images. 

![Figure 2. (a) AFM height image of MGP hydrogel, and (b) its corresponding phase image; (c) AFM height image obtained at the edge of MGP hydrogel; (d,e) TEM images of MGP hydrogel; (f) statistical analysis of the diameter of fibers. Conc. MGP is 1 wt %. Scale bars are 500 nm for (a,b,d,e), and 250 nm for (c).](image-url)
in Figure S8, it was strongly suggested that gel fibers reduced Au\textsuperscript{3+} and capped AuNPs in situ spontaneously within the network.\textsuperscript{31,32} This was rare that in situ formation of AuNPs occurred spontaneously in a phosphate-contained gel, nucleated by the nanofibers without external reductants.

UV−vis, XPS, ATR-FTIR, XRD, AFM, and TEM were utilized to gain insights into AuNPs in this hybrid gel. The composite MGP−AuNPs displayed a strong absorption band at approximately 540 nm (Figure S9), corresponding to its surface plasmon resonance band.\textsuperscript{33,34} Either change in MGP concentration or gel concentration with keeping the molar ratio of MGP/HAuCl\textsubscript{4} did not affect the particle size. Moreover, in XPS 84 and 88 eV were detected, which were assigned to 4f\textsubscript{7/2} and 4f\textsubscript{5/2} in the oxidation state of Au\textsuperscript{0} with a 4 eV spin−orbit splitting (Figure S10). ATR-FTIR showed that the bands for P=O (1319, 1213, and 1156 cm\textsuperscript{−1}) and P−O−C (1019 cm\textsuperscript{−1}) shifted slightly upon the formation of the MGP−AuNPs hybrid gel (Figure S11). In addition, XRD results in Figure S12 revealed the typical reflection peaks of Au at 38.19° (111), 44.39° (200), 64.58° (220), and 77.57° (311), respectively. AFM and TEM images revealed that the diameters of MGP−AuNPs was approximately 10−20 nm (Figure 3), and the atomic planes spacing in these nanocrystalline particles were clearly visible under high-resolution TEM (Figure S13). It should be pointed out that AuNPs were mainly deposited on fibers instead of gel cavities. This phenomenon was ascribed to ligand−metal interactions as stated by Stupp et al.\textsuperscript{35−38} Thus, we proposed a possible mechanism as follows: initially, Au\textsuperscript{3+} ions bound to phosphate groups on the nanofibers, and such synergetic binding effect made electrons in oxygen delocalization, which further promoted the hydrolysis of phosphoester bonds; subsequently, the resulting hydroxyl groups on GA skeletons were oxidized by Au\textsuperscript{3+} to carbonyl groups with the formation of Au atoms in situ; consequently, Au atoms coalesced to form clusters, which then grew to yield AuNPs on nanofibers.\textsuperscript{39−41} As no direct evidence for this proposed mechanism was obtained so far, a deeper study in a detailed mechanism will be explored in our future work.

The impact of AuNPs on the gel stability was determined by a rheological study. As shown in Figure S5, the G’ for MGP−AuNPs hybrid gel was around 10 times higher than that for mere MGP hydrogel. Such an improvement has ever been presented by Smith and co-workers in 2016,\textsuperscript{42} and it was suggested that AuNPs helped cross-link the gel fibers. Moreover, the immobilization of AuNPs on nanofibers prevented the uncontrolled aggregation of Au\textsuperscript{0} during spontaneous in situ reduction, and these AuNPs were kept stable for more than 3 months under ambient conditions.

Traditional depositing of AuNPs onto electrodes has some shortages, like the generation of bulk gold and the leakage of NPs.\textsuperscript{43−45} However, the hydrogel in this work could bind AuNPs onto the electrode effectively, while maintaining the stability and integrity of AuNPs, thus resulting in the formation of porous electrochemical surfaces. To this end, a preliminary exploration of MGP−AuNPs hybrid gel for the electrode modification was made. MGP−AuNPs hybrid gel was generated as described above, and any free Au\textsuperscript{3+} or AuNPs was removed by submerging the modified electrode in DI water for 3 h. Cyclic voltammetry was used to investigate the gel-catalyzed O\textsubscript{2} reduction, scanning in air between −0.4 and 1.0 V. As shown in Figure 4, in comparison with bare CPE and CPE modified with MGP hydrogel, a large reductive current was observed for CPE modified with MGP−AuNPs hybrid gel at

![Figure 3. (a,b) TEM images of MGP−AuNPs hybrid gel; (c) enlarged area of TEM image displayed in (b); (d,f) AFM height images of MGP−AuNPs hybrid gel; (e) corresponding AFM phase image of (d). White arrows point to AuNPs on the fibers. Scale bars are 1 μm for (a,d,e), 500 nm for (b,f), and 200 nm for (c).]

![Figure 4. Cyclic voltammetry of bare CPE (black) and CPE modified with MGP hydrogel in the absence (red) and presence (blue) of AuNPs.]

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the potential more negative than $-0.2 \text{ V versus AgCl/Ag (saturated KCl)}$, attributing to Au-catalyzed $O_2$ reduction. Apparently, AuNPs embedded in the hybrid gel enabled the communication with a carbon electrode.

Furthermore, the conductance of this hybrid gel was initially performed. In general, it is difficult to manipulate the supramolecular gels because of the mechanical weakness, thus MGP was mixed with polymeric agarose to create a more robust hydrogel, which was an effective strategy for making robust materials. Similarly, a heat–cool process led to the formation of MGP–agarose gel as shown in Figure S14. The AuNP formation in the MGP–agarose gel was wholly analogous to that in MGP hydrogel alone, and no AuNPs was observed in mere agarose hydrogel.

A mold was used to produce the hybrid hydrogels with the reproducible dimensions, which then were immersed in $Au^{3+}$ aqueous solution to yield AuNPs. As shown in Figure S15, graphite block electrodes were fixed to a plastic plate with a certain distance. After placing a block of gel between graphite rods, the conductance of MGP–agarose–AuNPs gel and MGP–agarose gel were determined. As shown in Figure 5, MGP–agarose–AuNPs gel exhibited the enhanced conductance in comparison with MGP–agarose gel. Given that AuNPs was around 10–20 nm and located on nanofibers instead of gel cavities, it was assumed that the increase in conductivity of MGP–agarose–AuNPs was arisen from a similar way as metalloproteins. In addition, the conductance increased significantly after the blocks of MGP–agarose–AuNPs gel were air-dried for 3 h with losing 40% water. Because the fibers in the gel state were fully solvated with far distance between each other, it limited the conductivity. It was proposed that the drying shortened the distance among AuNPs and consequently increased the network connectivity.

Besides gold salts, to test the feasibility of MGP hydrogel as an efficient leaching extraction agent, an aqueous solution containing $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Fe^{3+}$, $Pd^{2+}$, and $Au^{3+}$ was utilized as a leaching sample to diffuse into the hydrogel under ambient conditions for 2 days (metal chlorides, 8 mM, 1 mL; MGP 2 wt %). The supernatant fraction was analyzed by ICP-MS, and the results are shown in Figure 6a and Table S3. This hydrogel exhibited a stronger binding ability toward heavy metal ions, and the best extracted ones were those owning the high reductive potentials, which was caused by the reduction ability of fibers to heavy metal ions. Meanwhile, fibroblast 3T3L1 cells were cultured with MGP to investigate its cytotoxicity. Preliminary results showed that there was no obvious toxicity with the concentration up to 600 $\mu M$ using the CellTiter-Blue assay (Promega) (Figure 6b). As shown in Figure 6c–e, live cells were stained green and dead cells were in red color, indicating that the majority of 3T3L1 cells survived in presence of MGP (600 $\mu M$) for 24 h. However, cell viability and morphology were the only parameters examined in this work as preliminary suitability indicators of MGP hydrogel for converting heavy metals from water to metal NPs in situ spontaneously. Future study will aim to expand the other applications of MGP hydrogel.

**CONCLUSION**

In summary, we have synthesized an amphiphilic natural triterpenoid-tailored phosphate MGP, and it could form the stable hydrogel after a simple heat–cool process under ambient conditions. By extracting gold salt from water, AuNPs formed in situ spontaneously on gel fibers without external reductants rather than in gel cavities, probably because of the interactions between phosphate-functionalized nanofibers and Au ions. Moreover, the resulting hybrid gels exhibited attractive electrocatalytic and conductive properties. As an efficient leaching extraction agent, MGP hydrogel exhibited higher affinity toward heavy metals over other common metals and displayed good biocompatibility. This work not only provides a potential way in converting heavy metals to metal NPs spontaneously but also expands the application of nature product-based soft materials.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b03569.

Synthesis and characterization of MGP; gelation tests of MGP; morphologies of MGP gel under different concentrations; removal of DMSO in MGP gel; rheological tests; high-resolution TEM images of MGP hydrogel; driving forces and possible packing pattern of MGP hydrogel; redox reactions of MGP with $HAuCl_4$ in...
various solvents; AFM images for MGP in different solvents; UV–vis spectra of composite MGP–AuNPs; XPS spectra of MGP–AuNPs; ATR-FTIR spectra of MGP–AuNPs; XRD pattern of MGP–AuNPs; high-resolution TEM images of MGP–AuNPs; photographs of agarose and MGP–agarose gels in presence of H3AuCl6 and “in house” device for measuring the conductance of hybrid gel; extraction of metal ions by MGP hydrogel (PDF)

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**Notes**
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