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A facile method to *in situ* fabricate three dimensional gold nanoparticles micropatterns in a cell-resistant hydrogel

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Ming-Hao Yao, Jie Yang, Dong-Hui Zhao, Rui-Xue Xia, Rui-Mei Jin, Yuan-Di Zhao*, and Bo Liu*

A facile method for *in situ* fabrication of three-dimensional gold nanoparticles micropatterns in a cell-resistant polyethylene glycol hydrogel has been developed by combining photochemical synthesis of gold nanoparticles with photolithography technology. Gold nanoparticles micropatterns were further bio-modified with cell integrated polypeptide NcysBRGD based on gold-thiol bond to improve cell behaviors. Primary cell tests showed that NcysBRGD can enhance cell adhesion very well on the surface of gold nanoparticles micropatterns.

Micropatterning technology has received much attention in many fields, especially in cell biology studies, tissue engineering, biological assays, and drug screens.[1] Micropatterned surfaces provide a powerful way for precisely manipulating chemical compositions and topographical properties of the surface microenvironment to mimic the extracellular matrix (ECM) for investigating the interactions of cells and batteries,[2] and the associated fundamental researches which might be very crucial for the development of tissue engineering and reconstruction.[3] Up to date, significant development for the regulation of spatio-temporal distribution of inorganic particles or biomolecules in biomaterials has been made. However, most micropatterning techniques are applicable only to two-dimensional (2D) patterning.[4] For example, Ding group has proposed a perfect method to construct 2D gold micropatterns on the surface of PEG hydrogel by conventional lift-off photolithography technique.[5] Compared with fabrication of 2D micropatterns, three-dimensional (3D) micropatterns can better mimic the microenvironment of native ECM. Dramatic differences between cells migrating on 2D surface and that within 3D matrices have been observed.[6]

To investigate tissue complexity and develop clinically relevant tissue, recent focus has been transferred to techniques for controlling the 3D arrangement of inorganic particles, biomolecules, and cells at microscale.[4a] Various 3D microfabrication techniques have been developed, such as microfluidic patterning, sequential photolithographic patterning, electrochemical deposition, soft-lithographic patterning, and 3D printing.[7] However, these micropatterning techniques are serially patterned in the *z* direction to form the final 3D structures, which generally suffers from the loss of fidelity in *z* direction during the iterative procedures.[8] In addition, the complex and time-consuming operations of these layer-by-layer methods are still limited.

Herein, a facile and one-step method of *in situ* preparation of 3D gold nanoparticles (GNPs) micropatterns in the cell-resistant polyethylene glycol (PEG) hydrogel has been developed by combining photochemical synthesis of GNPs with photolithography technology (Scheme 1a). This technique is effective in cost and time. GNPs can be synthesized in seconds to minutes under 365 nm UV light throughout the PEG hydrogel substrate. Combining with photolithography technology, 3D GNPs micropatterns throughout the PEG hydrogel substrate could be *in situ* achieved within minutes. A basic polypeptide containing a cysteine and a cell-binding domain arginine-glycine-aspartic acid (RGD) was incorporated in 3D GNPs micropatterns by normal gold-thiol bond to promote cell adhesion. The sequence of the designed polypeptide was shown in Scheme 1b. The micromorphology of GNPs and micropatterns was studied, and preliminary cell tests were also performed. Thus, the technology reported here may be used for tissue engineering and as a potentially viable tool for fundamental studies of cell behaviors in 3D microenvironments.

[a] *Britton Chance Center for Biomedical Photonics at Wuhan National Laboratory for Optoelectronics - Hubei Bioinformatics & Molecular Imaging Key Laboratory, Department of Biomedical Engineering, College of Life Science and Technology, Huazhong University of Science and Technology, Hubei, Wuhan 430074, P. R. China*

[b] *E-mail: zydi@mail.hust.edu.cn (Y.-D. Zhao), lbyang@mail.hust.edu.cn (B. Liu); Fax: (+) 86 27-8779-2202*

† Electronic Supplementary Information (ESI) available: Experimental details. See DOI: 10.1039/c000000x/

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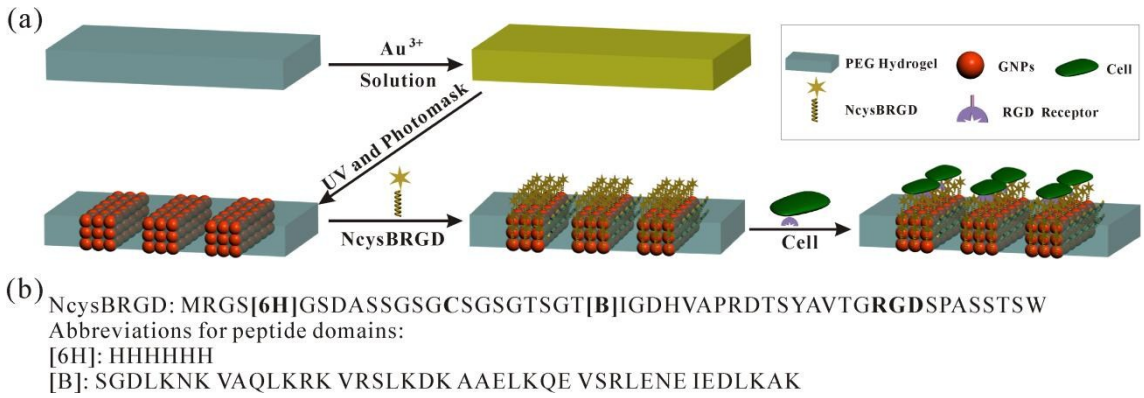

Scheme 1 (a) Schematic illustration of the *in situ* fabricating 3D polypeptide-modified GNPs micropatterns by the technology of photochemistry and photolithography. (b) The sequence of polypeptide NcysBRGD used in the study.

PEG was chosen as substrate to prepare micropatterns because of its excellent advantages of biocompatibility, non-toxicity, non-fouling in complex environments, and biologically inert due to hydrophilicity and charge neutrality. In addition, the property of PEG, preventing unspecific protein adsorption and undesired cell attachment, also makes it a perfect biomaterial as the cell-resistant hydrogel substrate for micropatterns.[9] Lots of noble metal could be used as the building blocks for micropatterning for biological applications, such as gold and silver. We use gold in our experiment because gold is the well studied, most used and reported material. GNPs are well known as cell adhesion materials and commonly used as a scaffold to immobilize biological cues for mediating cell adhesion, which have been frequently used to study cell behaviors.[10] Different size of unprotected aqueous GNPs have been synthesized in seconds to minutes by photochemical decomposition of I-2959 under 365 nm UV light condition.[11] Therefore, the synthesis of GNPs could be controlled by combining photochemical synthesis with photolithography technology, which will be convenient for *in situ* fabricating 3D GNPs micropatterns throughout the cell-resistance PEG hydrogel substrate. The method of fabricating polypeptide-modified 3D GNPs micropatterns for biological applications is shown in Scheme 1. The PEG hydrogel substrate was prepared by photopolymerization of polyethylene glycol diacrylate (PEGDA, molecular weight: 2 kDa). The ready-made PEG hydrogel substrate was immersed into 1% I-2959 solution containing 2.4 mM $HAuCl_4.4H_2O$ for several minutes to allow Au^{3+} seeping into hydrogel. Combining with photolithography, GNPs micropatterns were photochemically fabricated under UV (365 nm, 500 W) light. Finally, a leucine zipper polypeptide-engineered containing a cysteine on the N-terminal and a cell-binding motif RGD on the C-terminal was immobilized on the surface of GNPs in 3D GNPs micropatterns by normal gold-thiol bond to promote cell adhesion. The flexibility of recombinant DNA technology allows preparation of polypeptides with precise structure at the molecular level.[12] Therefore, sequences of interest, such as binding domains and enzyme cleavage sites can be incorporated into engineered polypeptides. This method is very brief and practical, and the 3D micropattens can be accomplished very quickly.

To better understand the photochemical synthesis of GNPs in I-2959 solution, experiments without any PEGDA were performed firstly. Different factor including original concentration of Au^{3+} and I-2959%, irradiation time, and irradiation intensity was investigated. GNPs were synthesized as shown in Fig. 1a. Compared a with b, b with c, c with d, d with e, we could conclude that more original concentration of Au^{3+} and I-2959%, longer UV irradiation time, larger UV irradiation intensity, and more GNPs will be got in solution. Therefore, the GNPs synthesized by photochemical method could be easily tuned through these factors. In addition, the effect of the $PEGDA_{2k}$ polymer on GNPs formation in solution was also investigated. GNPs were photochemically prepared in ultrapure water, 0.2 M/L CTAB, and 1% w/v $PEGDA_{2k}$ solution containing 1% w/v I-2959, respectively. The size and morphology of GNPs were contrasted and analyzed by TEM and UV-vis absorption spectra (Fig. 1b-d). As shown in Fig. 1b, almost all GNPs were aggregated, which may be due to the lack of stabilizer to disperse GNPs. While no macroscopic aggregation of both GNPs-CTAB (GNPs photochemically prepared in CTAB solution, Fig. 1c) and GNPs-PEGDA (GNPs photochemically prepared in PEGDA solution, Fig. 1d) were observed, and the sizes of GNPs were about 30 nm. The reason that no aggregations were observed in GNPs-PEGDA may be caused by the formation of a layer of PEG nanogel on the surface of GNPs which acts as dispersant through the exclusion between PEG. Another possible reason may be the formation of some little PEG nanogels in solution which prevent the interaction among GNPs. The maximum absorbance peak of GNPs-nothing (GNPs photochemically prepared in ultrapure water, Fig. 1b) , GNPs-CTAB, and GNPs-PEGDA was 564 nm, 526 nm, and 527 nm (Fig. 1e), respectively, suggesting that GNPs-PEGDA and GNPs-CTAB presented a well dispersity, and GNPs-nothing showed a macroscopic aggregation, which is consistent with the result of TEM. In addition, the peak width at half height of GNPs-PEGDA is larger than that of GNPs-CTAB, indicating that the size distribution of GNPs-CTAB is better than that of GNPs-PEGDA. These results suggest that PEGDA or PEGDA hydrogel can avoid GNPs aggregation.

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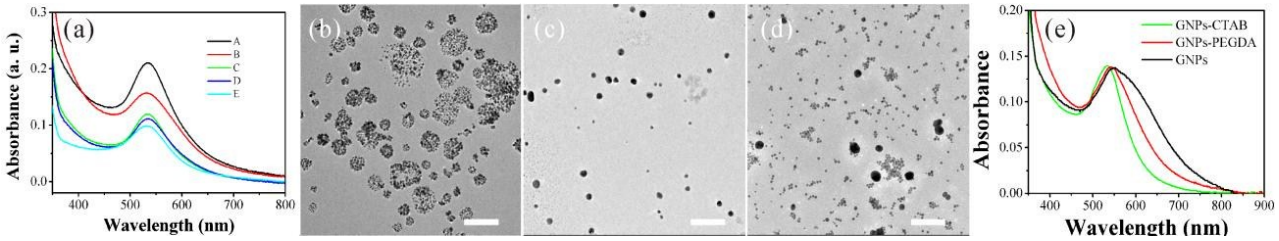

Fig. 1 The UV-vis absorption spectra of GNPs synthesized in the condition of A: 2.4 mM Au^{3+}, 1% (w/v) I-2959%, and 60 seconds; B: 1.2 mM Au^{3+}, 1% (w/v) I-2959%, 60 seconds, and 500 W; C: 1.2 mM Au^{3+}, 1% (w/v) I-2959%, 30 seconds, and 500 W; D: 1.2 mM Au^{3+}, 0.5% (w/v) I-2959%, 30 seconds, and 500 W; E: 1.2 mM Au^{3+}, 0.5% (w/v) I-2959%, 30 seconds, and 300 W (a). TEM images of GNPs photochemically prepared in ultrapure water (b), 0.2 M/L CTAB (c), and 1% w/v $PEGDA_{2k}$ (d) containing 1% w/v I-2959, and the UV-vis absorption spectra of GNPs, GNPs-CTAB, and GNPs-PEGDA (e). Scale bars are 100 nm.

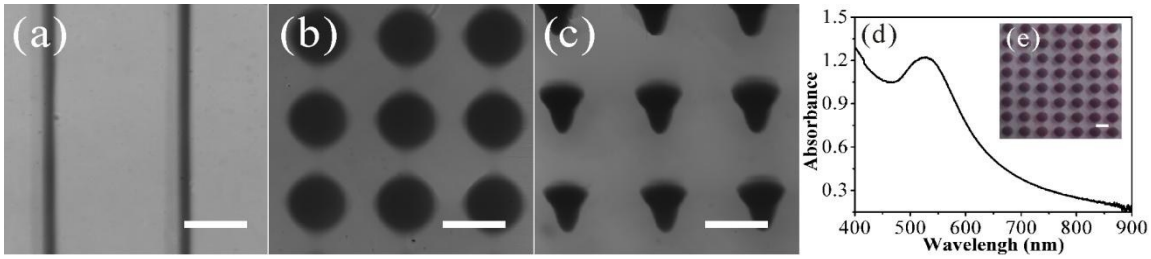

Fig. 2 Optical images of 3D GNPs micropatterns fabricated with different photomasks, line (a), circle (b), and thumbtack (c); and UV-vis absorption spectrum of 3D GNPs micropatterns (e). Scale bars are 200 μm.

By changing the pre-designed photolithography masks, various shapes of micropatterns (such as circle, line, and thumbtack) throughout hydrogel substrate can be easily obtained in minutes under 365 nm UV light. 3D GNPs micropatterns photolitographically fabricated through the photomasks having stripe, circle, and thumbtack patterns are shown in Fig. 2. The GNPs micropatterns are relatively regular. The diameter and width of micropatterns, and the space between two patterns were easy to be regulated by designing different photomasks. After exposure to the 365 nm UV light for several minutes, the color within UV light exposed areas obviously changed from yellow to red (Fig. 2e), indicating that Au^{3+} was reduced to form GNPs by photochemical decomposition of I-2959.[13] The UV-vis absorption spectrum of 3D GNPs micropatterns showed a maximum absorbance at around 520 nm (Fig. 2d), which was close to the reported surface plasmon resonance of GNPs.[14] This result verified that the GNPs and 3D GNPs micropatterns in the PEGDA hydrogel have been synthesized.

The surface morphology of GNPs micropatterned hydrogel was observed by SEM (Nova NanoSEM450). Carbon element was used to spray on samples for increasing the electrical conductivity of samples. Micropatterned stripes could be seen clearly in Fig. 3A. In Fig. 3A1 and 3A2, the EDXS (Energy Dispersive X-ray Spectrometer) results showed that the Wt% of Au element in micropatterned area (4.61%) was much higher than unpatterned region (0.87%), which confirmed the successful synthesis of GNPs in patterned place. To observe the distinction of microcosmic morphology between micropatterned and unpatterned place, we amplified the interface of them. As can be seen in Fig. 3B, the surface morphology of GNPs micropatterned region (b1) displayed an obvious difference with that of unpatterned area (b2). There were lots of bulges in the micropatterned area, while nothing could be seen in the unpatterned region. These bulges should be caused by the formation of GNPs. UV-vis absorption spectrum of 3D GNPs micropattern (Fig. 1e) also confirmed the formation of GNPs in these areas. The sizes of the bulges are about 1 to 5 μm (Fig. 3B11), which are much larger than those of single GNP. This result suggests that each bulge contains lots of GNPs. However, the peak of UV-vis absorption spectrum of 3D GNPs micropatterns is about 520 nm (Fig. 1e), indicating that the fabricated GNPs are not aggregated in the PEGDA hydrogel. Therefore, the GNPs in the 3D micropatterned area should be uniformly dispersed in the bulges, which may be related with the PEGDA hydrogel. In addition, micropatterned hydrogels of different concentration PEGDA (10% and 20% w/v) and original Au^{3+} (1.2 and 2.4 mM)

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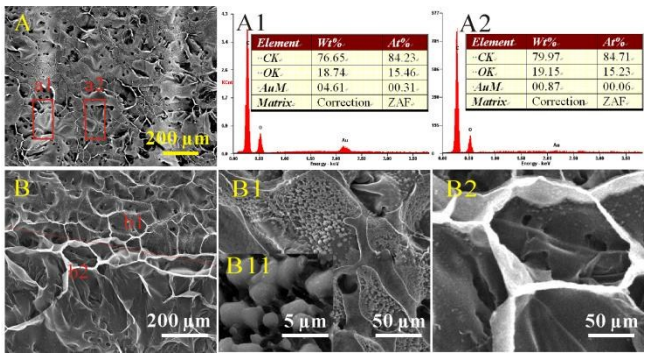

Fig. 3 SEM image of stripe GNPs-micropatterns (A); EDXS analyse result of stripe area a1 (A1) and no stripe area a2 (A2); SEM image of the interface (B) of micropatterned area and unpatterned area; the amplification of micropatterned area b1 (B1, B11) and unpatterned area b2 (B2).

were prepared and observed by SEM (Fig. S1). The pore size of 10% PEGDA hydrogel is over 50 μm, which is larger than that of 20% PEGDA hydrogel (about 50 μm). The amount of GNPs in the PEGDA hydrogel with 2.4 mM original Au^{3+} is more than that of 1.2 mM original Au^{3+}. These results demonstrated that both of the pore size of hydrogel and the amount of GNPs in hydrogel could be well tuned.

Fabrication of 3D micropattern can better mimic the natural 3D cell microenvironment with soluble and physical cues.[15] Optical and SEM image of the vertical section of 3D micropattern shows that GNPs micropattern presents throughout the PEGDA hydrogel from top to bottom (Fig. 4). The result indicates that this GNPs micropattern is a kind of 3D micropattern, and the formation of GNPs presents not only on the surface of PEGDA hydrogel but also within the hydrogel. 2D micropattern is perfect for investigating the cell behaviors on the surface of 2D materials, which is not enough to mimic the cell behaviors in a complex 3D microenvironment due to the different cell behaviors in 2D and 3D conditions. Therefore, the 3D micropatterns can provide a perfect platform for investigating the cell-factor interactions in the 3D microenvironment for biological applications.

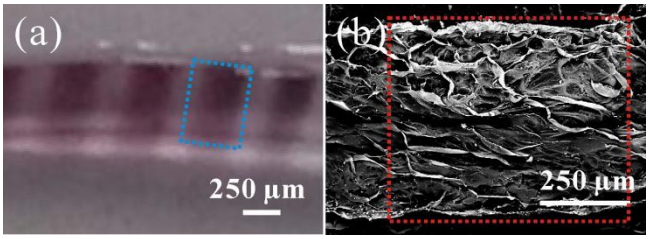

Fig. 4 Optical (a) and SEM (b) image of the vertical section of 3D GNPs micropatterns throughout PEGDA hydrogel.

To study the cell-adhesion-resistant ability on the GNPs micropatterned PEGDA hydrogel and assess the biocompatibility of this hybrid material, preliminary cell culture had been investigated. Cell culture on the PEGDA hydrogel and PEGDA-GNPs hydrogel (PEGDA hydrogel containing GNPs) as control groups were tested. After 2 hours, almost no cells adhered on the surface of the PEGDA hydrogel (Fig. 5a), which is consistent with previous researches that PEG hydrogel was cell-resistant. Many cells could adhere on the surface of PEGDA-GNPs hydrogel (Fig. 5b). However, most of these cells presented round shape. Cell adhesion on the PEGDA-GNPs hydrogel may be caused by the cell-adhesion ability of the GNPs. This result indicates that many GNPs exposure on the surface of hydrogel. In order to address the challenge of cell adhesion, a polypeptide NcysBRGD containing a cell-binding tripeptide RGD was used to immobilize on the surface of GNPs by the gold-thiol bond within the PEGDA hydrogel. As we know, the RGD sequence could specially combine with 11 kinds of integrins and effectively promote cell adhesion in biomaterials.[16] In Fig. 5d, many cells efficiently attached and spread on the surface of GNPs unpatterned hydrogel modified with NcysBRGD polypeptide. In order to further confirm that it was the synergistic effect of GNPs and NcysBRGD to achieve this, PEGDA hydrogel without GNPs was prepared, modified with NcysBRGD solution, and cultured with HeLa cells. Few cells were observed on the surface of PEGDA hydrogel without GNPs modified with NcysBRGD (Fig. 5c). This result may be due to the lack of GNPs to bind NcysBRGD for cell adhesion. As expected, the cells efficiently attached and spread on the NcysBRGD-modified micropatterned areas (Fig. 5e), while few cells were observed in the unpatterned areas. This result indicates that the NcysBRGD can be immobilized on the GNPs within the PEGDA hydrogel to promote cell adhesion. Two dimensional adhesion studies have served as a valuable first to understanding cell motility, specific cell-materials interactions, and cell-cell interactions. However, cell fate on the 2D and within 3D microenvironment is different. Therefore, fabrication of 3D micropatterns to mimic native ECM is particularly important. This kind of 3D GNPs micropattern will provide a platform for biological applications, such as mimicking tissue complexity and developing relevant tissues.

In conclusion, this communication had presented a very brief method for generating a 3D cell-adhesion GNPs micropattern throughout the cell-resistant PEGDA hydrogel substrate by combining photolithography technology with the photochemical synthesis of GNPs *in situ*. 3D GNPs micropattern throughout hydrogel can be achieved within minutes. The pore size of hydrogel and the amount of GNPs within hydrogel could be easily regulated. The polypeptide fusion of bioactive ligands of interest was immobilized on the surface of GNPs within 3D micropattern to promote cell adhesion. Therefore, this kind of 3D micropattern technology will provide a new tool for studying cell-biomaterial interaction under 3D microenvironments.

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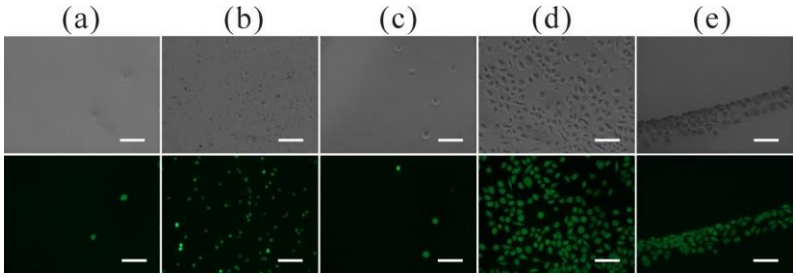

Fig. 5 Adhesion of HeLa cells on the PEGDA hydrogel (a), PEGDA-GNPs hydrogel (b), NcysBRGD-modified PEGDA hydrogel without GNPs (c), NcysBRGD-modified GNPs unpatterned hydrogel (d), NcysBRGD-modified GNPs micropattern hydrogel (e). All images (over: bright-field; under: fluorescence) were acquired after the samples were stained with calcein AM and Ethidium homodimer-1. The scale bars are 100 μm.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 81271616, 81471697), the Foundation for Innovative Research Groups of the NNSFC (Grant No. 61121004), the Natural Science Foundation of Hubei Province (2014CFB1010), the Key Technology R&D Program of Hubei Province (2014BBB003) and Yellow Crane Talent (Science & Technology) Program of Wuhan City, 61121004), the Open Research Fund of Collaborative innovation center of modern bio-manufacture, Anhui University, and the Fundamental Research Funds for the Central Universities (Hust, 2015TS088).We also thank the facility support of the Analytical and Testing Center (HUST) for the help of measurement.

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Graphical Abstract

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