DOI: 10.1002/cjoc.201600813

Supramolecular Hydrogels of Indole-Capped Short Peptides as Vaccine Adjuvants

Zhongyan Wang,[a,b] Yanbin Cai,[a,b] Linan Yi,[a,b] Jie Gao,*[,a] and Zhimou Yang*[,a,b]

[a] *State Key Laboratory of Medicinal Chemical Biology*, *Nankai University*, *Tianjin 300071*, *China*
[b] *College of Life Sciences*, *Nankai University*, *Tianjin 300071*, *China*

Many materials as immune adjuvant are researched to help raise immnogenicity of subunit vaccines. Among them, peptide-based hydrogels are gradually coming into notice because of their application in drugs delivery, cancer cell inhibition, vaccine adjuvants and detection of important analytes. In this work, we introduced a novel aromatic capping group based on indole to construct short peptide-based supramolecular hydrogelators Indol-GFFY and Indol-G^{D}F^{D}F^{D}Y and demonstrated their potential applications as vaccine adjuvants.

Keywords self-assembly, hydrogel, vaccine adjuvant

Introduction

Subunit vaccines are being widely developed owing to their well-defined structure at the molecular level, excellent safety properties, and ease of production. However, relative to live vaccines, subunit vaccines do not raise strong immune responses.[1] Subunit vaccines are therefore formulated with adjuvants to stimulate more potent immune responses. Many materials have been demonstrated to be promising vaccine adjuvants, including hydrogels and nanomaterials (*e.g.*, liposomes and mesoporous silica nanoparticles).[2] Among these materials, hydrogels are relatively easy to handle because antigens can simply be mixed with hydrogels before use. Recently, Collier *et al.* conducted pioneering work whereby supramolecular peptide hydrogels were used as immune adjuvants.[3] They demonstrated that nanofibres composed of bioconjugates of self-assembling peptides and peptide/protein antigens could raise strong humoral immune responses in a long-lasting manner.[4] Our group has also demonstrated that enzyme-catalysed short peptide-based hydrogels can raise strong immune responses when physically mixed with HIV DNA or protein (ovalbumin (OVA)) antigens.[5] These results suggested the great promise of supramolecular hydrogels as vaccine adjuvants.

Peptide-based hydrogels have shown great promise for applications in drug delivery,[6] cancer cell inhibition,[7] and detecting important analytes.[8] Among peptide-based hydrogelators, those based on short peptides are easier to design and synthesize. To obtain short peptide-based hydrogelators, peptides are typically capped with an appropriate aromatic group, such as fluoren-9-ylmethyloxycarbonyl (Fmoc), naphthalene (Nap), phenothiazine (PTZ) or indole (Indol).[9] We chose to test whether hydrogels formed by indole-capped short peptides could be potential vaccine adjuvants because indole is commonly found in a wide variety of natural products, including the amino acid tryptophan, and is highly biocompatible.[9l]

Experimental

Materials

2-Cl-Trityl chloride resin (1.2 mmol/g) was obtain from Nankai University Resin Co., Ltd. *O*-Benzotriazol-1-yl-*N*,*N*,*N*',*N*'-tetramehtyl uronium hexafluorophosphate (HBTU) and Fmoc-amino acids were bought from GL Biochem (Shanghai). Chemical regents and solvents were used as received from commercial sources. Commercially available reagents were used without further purification, unless noted otherwise. RMPI 1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco Corporation. EndoFit Ovalbumin (endotoxins＜1 EU/mg) was purchased from InvivoGen (CA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, and IgG2b were obtained from Southern Biotechnologies (AL, USA). Mouse IL-5 and IFN-γ ELISA kits were obtained from Biolegend (CA, USA). Six- to eight-week-old female C57BL/6J mice were purchased from Academy of Military Medical Science (Beijing, China), and maintained in specific pathogen-free conditions in the animal facility at the Nankai University, Tianjin, China.

* E-mail: yangzm@nankai.edu.cn, chemgaojie@nankai.edu.cn; Tel.: 0086-022-23502875; Fax: 0086-022-23507760
Received November 21, 2016; accepted January 23, 2017; published online XXXX, 2017.
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cjoc.201600813 or from the author.

General procedure

^{1}H NMR (Bruker ARS 400) and HR-MS (Agilent 6520 Q-TOF LC/MS) were used to characterize the compounds. TEM (JEM100CXII) was performed at the Tecnai G2 F20 system, operating at 100 kV. Reology (TA instrument) test was done on an AR 2000ex system, 40 mm parallel plates were used during the experiment at the gap of 500 μm. Fluorescence spectrum was gained by BioTek (Synergy 4). Circular dichroism (CD) spectrum was obtained by a BioLogic Synergy 4.

Synthesis and characterizations

1-Indolepropionic acid synthesis As shown in Scheme S1 (Supporting Information), indole (21.9 mg, 2.56 mmol), potassium hydroxide (171 mg, 2.61 mmol, 85%, freshly ground), and sodium iodide (39 mg, 0.26 mmol) were dissolved under argon in 10 mL dry DMF. After 5 min stirring at room temperature, the ethyl 3-bromopropionate (7.7 mmol) was added dropwise. The reaction mixture was heated at 50 ℃ for 42 h. After the reaction mixture was cooled back to room temperature for 30 min, it was diluted with DCM (15 mL) and washed with water (10 mL×3). The combined organic layers were dried over magnesium sulfate, filtered, and rotary-evaporated to give a yellow liquid. The crude product was purified on a flash silica column with EtOAc/hexanes (4% to 8% to 15%) as eluents to give the indolepropionic ethyl ester, which was then hydrolyzed in EtOH/MeOH/KOH (25∶25∶8, 116 mL) at 60 ℃ for 30 min. The reaction mixture was poured into 100 mL water and acidified by slow addition of 10% citric acid until the salt was converted completely to the free acid. The resulting crystal was filtered and washed with cold water. The crude acid was recrysallized from ethanol to afford the final product.

Synthesis of peptides Both Indol-Gly-Phe-Phe-Tyr-OH (Indol-GFFY, **1**) and Indol-Gly-*D*-Phe-*D*-Phe-*D*-Tyr-OH (Indol-G^{D}F^{D}F^{D}Y, **2**) were prepared by standard solid phase peptide synthesis (SPPS)[10] by using 2-chlorotrityl chloride resin and the corresponding *N*-Fmoc protected amino acids with side chains properly protected. Firstly, the C-terminal of the first amino acid was conjugated on the resin. Anhydrous *N*,*N*'-dimethyl formamide (DMF) containing 20% piperidine was used to remove Fmoc protected group. To couple the next amino acid to the free amino group, HBTU was used as coupling reagent. Peptides chain was extended according to the standard SPPS protocol. After the 1-indolepropionic acid coupling the glycine, 5 times DMF wash followed by 5 times DCM wash were used to remove excessive reagents. Lastly, 95% TFA containing 2.5% H_2O and 2.5% TIS was used to cleave peptides derivative from resin and the mixture was filtered. Ice cold diethylether was poured into filtrate performed by rotary evaporation. The precipitate was centrifuged for 5 min at 5000 r/min speed. The solid was dried by vacuum pump to gain resulting compounds. We then obtained the pure compounds by reverse phase high performance liquid chromatography (HPLC).

Characterization of Indol-GFFY ^{1}H NMR (400 MHz, DMSO) δ: 9.22 (s, 1H), 8.18 (d, J=24.5 Hz, 3H), 8.00 (d, J=8.5 Hz, 1H), 7.51−7.44 (m, 2H), 7.20 (d, J=27.3 Hz, 11H), 7.02 (s, 3H), 6.66 (d, J=8.0 Hz, 2H), 6.37 (s, 1H), 4.52 (d, J=27.9 Hz, 2H), 4.36 (d, J=3.7 Hz, 2H), 3.65 (s, 1H), 3.54 (s, 1H), 3.02−2.91 (m, 4H), 2.84−2.78 (m, 2H), 2.67−2.59 (m, 4H). MS: calcd M =703.78, obsvd. $(M+H)^+$=704.3085.

Characterization of Indol-G^{D}F^{D}F^{D}Y ^{1}H NMR (400 MHz, DMSO) δ: 9.22 (s, 1H), 8.19 (dd, J=29.6, 13.3 Hz, 3H), 8.00 (d, J=8.4 Hz, 1H), 7.52−7.42 (m, 2H), 7.23 (t, J=24.1 Hz, 11H), 7.03 (d, J=7.8 Hz, 3H), 6.66 (d, J=8.4 Hz, 2H), 6.37 (s, 1H), 4.52 (d, J=27.9 Hz, 2H), 4.36 (d, J=6.4 Hz, 2H), 3.67 (d, J=10.2 Hz, 1H), 3.54 (s, 1H), 2.98 (dd, J=32.9, 10.6 Hz, 4H), 2.81 (d, J=18.6 Hz, 2H), 2.70−2.56 (m, 4H). MS: calcd M=703.78, obsvd. $(M+H)^+$=704.3077.

Hydrogel formation and characterizations

Preparation of peptide hydrogels Compound **1** or **2** was dispersed in sterile endotoxin-free PBS buffer (pH=7.4) at a final concentration of 0.2 wt% with sodium carbonate to adjust the final pH at 7.4. The suspension was then heated to 80 ℃ in order to dissolve the compound completely, then the hot solution was cooled back to room temperature (25 ℃). The hydrogel formed after about 60 min at room temperature.

Rheology Rheology test was carried out on an AR 2000ex (TA instrument) system, 40 mm parallel plates were used during the experiment at the gap of 500 μm. For the dynamic time sweep, the solution of compounds was directly transferred to the rheometer and it was conducted at the frequency of 1 rad/s and the strain of 1% immediately. The gels were also characterized by the mode of dynamic frequency sweep in the region of 0.1−100 rad/s at the strain of 1%. All samples were tested at temperature of 37 ℃.

TEM sample preparation Negative staining technique was used to observe the TEM images of micro structure in hydrogels. Firstly, 10 μL hydrogels were loaded on the 400 mesh copper grids coated with continuous thick carbon film, dd-water was then used to rinse grid twice. After rinsing, the grids containing samples were stained with 2 % uranyl acetate. Finally the grids were allowed to dry in air.

Preparation of peptide/OVA gels 20 μL OVA stock solution (10 mg/mL) was added and mixed with 980 μL hydrogel with pipette at room temperature (25 ℃). Then the OVA was dispersed in the hydrogel evenly by vortex and the mixture became a sticky liquid. The final concentration of OVA in hydrogel was 200 μg/mL. After about 60 min, the sticky liquid formed hydrogel again and became stable. When conducting immunization, first shakes the OVA/hydrogel and then aspirates the hydrogel with a syringe.

Immunizations For *in vivo* immune evaluation, female C57BL/6J mice were randomly divided into 4

groups and each group contained 6 mice. 4 groups mice were each given 100 μL subcutaneous injections (0.2 wt% supramolecular hydrogels composed of 20 μg OVA vaccine, 100 μL PBS with 20 μg OVA and 20 μg OVA with 25 times alum, respectively). All immunizations were given 14 days apart. Serum was collected at 7 d after the second immunization for the antibody detection.

Determination of antibody titers OVA-specific antibody responses in mice were examined by ELISA. 96-well ELISA plates were coated with 10 μg/mL OVA antigen and stored at 4 ℃ overnight. After three washes with PBST (PBS buffer containing 0.05% tween 20), the plates were blocked by 1% BSA buffer (1% BSA in PBST solution) for 1 h at room temperature. Individual antisera were serially diluted in the 1% BSA buffer and incubated in the wells for 2 h at 37 ℃. After five washes with PBST, the wells were incubated with goat anti-mouse IgG horseradish peroxidase for 1 h at 37 ℃. After washing, antibody binding was assessed by adding 100 μL 3,3',5,5'-tetramethyl-benzidine peroxidase substrate to each well. The substrate reaction was terminated by adding 50 μL of 2 mol•L^{-1} H_2SO_4. Antibody isotypes were determined similarly using goat anti-mouse IgG1, IgG2a and IgG2b horseradish peroxidase. The plates were then read by an ELISA reader at an optical density of 450 nm. Antibody titers were calculated as the reciprocal serum dilution giving O.D. readings＞0.1 standard deviations above background levels as calculated using PBS at the same dilutions.

Splenocytes cytokine production At 7 days after the second immunization, splenocytes (5×106 cells/ml) were collected and seeded in 24-well plates, and then were re-treated with soluble OVA (50 μg/mL) at 37 ℃ for 96 h. The production of IFN-γ and IL-5 in cell culture supernatants was detected by using ELISA kit (Biologend, San Diego, CA, USA).

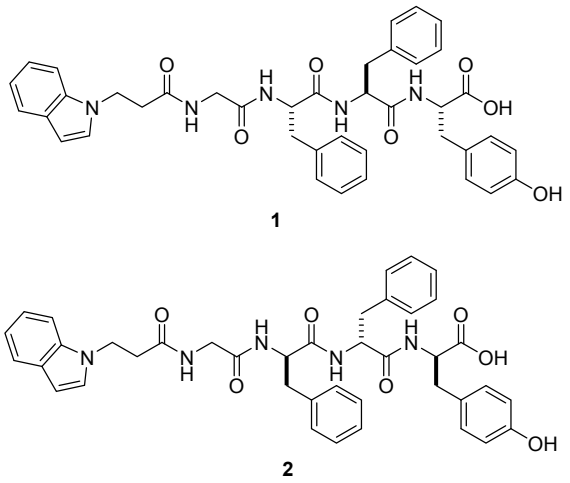
Cytotoxicity assays Biocompatibility of *L*-gel *D*-gel was evaluated by an MTT assay. The RAW 264.7 cells were seeded in 96-well plates at a density of 5000 cells per well with medium volume of 100 μL. After incubation for 24 h, 100 μL fresh medium containing a serial of concentrations of two gels was added. 72 h later, 10 μL MTT (5 mg/mL) was added into cells. After 4 h, DMSO was added to dissolve the formazan crystals. The optical density of the solutions at 597 nm was measured by a microplate reader. Meanwhile, cells without any treatment were used as the control. The percent of cell viability was calculated according to the following formula:

The percent of cell viability＝$OD_{sample}/OD_{control}$×100%.

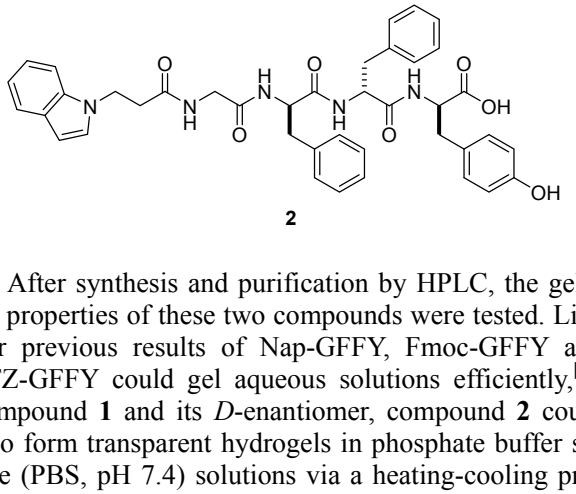
Results and Discussion

We designed two possible gelators (Indol-GFFY (**1**) and Indol-G^{D}F^{D}F^{D}Y (**2**) in Scheme 1) based on short peptide of GFFY, because many compounds with dipeptide FF or tripeptide FFY had excellent self-assembling and gelation properties.[11]

Scheme 1 The chemical structures of possible hydrogelators, Indol-GFFY (**1**) and Indol-G^{D}F^{D}F^{D}Y (**2**)



1



2

After synthesis and purification by HPLC, the gelatin properties of these two compounds were tested. Like our previous results of Nap-GFFY, Fmoc-GFFY and PTZ-GFFY could gel aqueous solutions efficiently,[12] compound **1** and its *D*-enantiomer, compound **2** could also form transparent hydrogels in phosphate buffer saline (PBS, pH 7.4) solutions via a heating-cooling process (Figure 1A, *L*-gel and *D*-gel, respectively) in 60 min. The minimum gelation concentration (MGC) for both compounds **1** and **2** was about 1.2 mg/mL.

We then characterized *L*-gel, *D*-gel, *L*-gel/OVA and *D*-gel/OVA by a rheometer and transmission electron microscopy (TEM). As shown in Figure 1E, both gels exhibited weak frequency dependences at the range of 0.1－100 rad/s. The elasticity (*G*' or storage modulus) value of both gels was about 10 times bigger than their corresponding viscosity (*G*'' or loss modulus) value, which means they were all true hydrogels. The *G*' value of all gels was more than 1000 Pa. These observations implied medium mechanical properties of all gels. However, the incorporation of OVA in gels made the gels slightly stronger. We observed flexible and uniform nanofibers in *L*-gel, *D*-gel, *L*-gel/OVA and *D*-gel/OVA (Figures 2A, 2B, 2C and 2D) and these flexible nanofibers entangled with each other to form three dimensional networks for hydrogelation. The diameter of nanofibers in *L*-gel and *D*-gel was similar and it was about 15－25 nm, and the incorporation of OVA in gels made the size of nanofibers slightly bigger (30－35 nm), which was likely the reason for the stronger mechanical properties of gels containing OVA.

In order to understand the molecular arrangement of peptides in nanofibers, we collected circular dichroism (CD) and emission spectra of solutions and gels. As shown in Figure 3A, we observed a positive peak near 194 nm ($\pi\pi^*$ transition) and negative bands at 208 nm

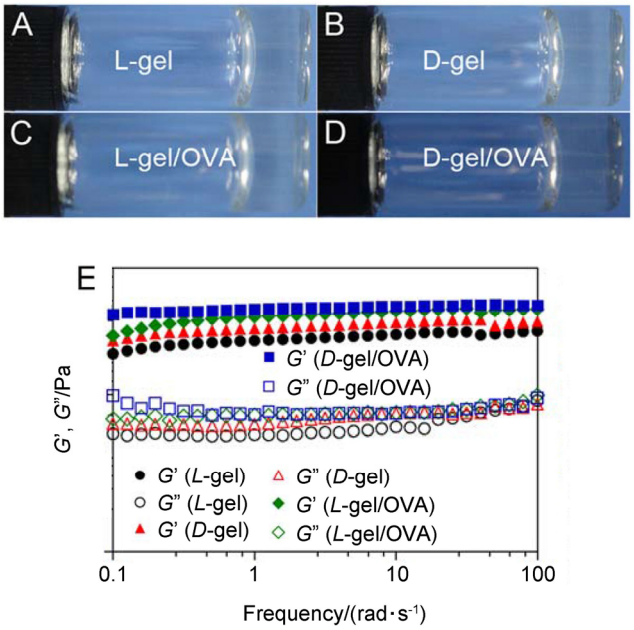


Figure 1 Optical images of (A) *L*-gel containing 0.2 wt% of **1**, (B) *D*-gel containing 0.2 wt% of **2**, (C) *L*-gel containing OVA (*L*-gel/OVA) and (D) *D*-gel containing OVA (*D*-gel/OVA). (E) Dynamic frequency sweep of *L*-gel, *D*-gel, *L*-gel/OVA and *D*-gel/OVA at 37 ℃. (For rheological characterization, three samples were tested for each group, one of the results was shown in this figure and the rest were shown in Supporting Information.)

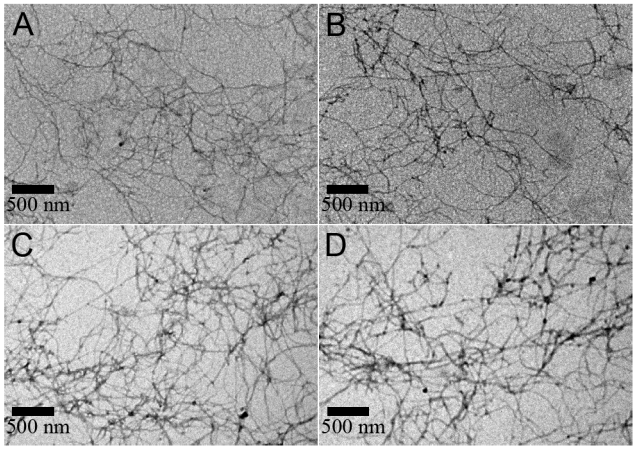


Figure 2 TEM image of (A) *L*-gel, (B) *D*-gel, (C) *L*-gel/OVA and (D) *D*-gel/OVA.

(nπ* transition) from *L*-gel, suggesting that compound **1** adopted the *β*-sheet conformation in the gel.[13] We did not observe distinct peaks at wavelength longer than 250 nm, suggesting that the indole moiety failed to adopt helical arrangement. The CD spectrum pattern from *L*-gel was similar to those from gels of Nap-Gly-Phe-Phe-Tyr-OH (Nap-GFFY) and Fmoc-Gly-Phe-Phe-Tyr-OH (Fmoc-GFFY), but slightly different from that from gel of PTZ-Gly-Phe-Phe-Tyr-OH (PTZ-GFFY) that possessed a negative peak at about 254 nm. The MGC value of compound **1** was therefore similar to those of both Nap-GFFY and Fmoc-GFFY (0.08—0.1

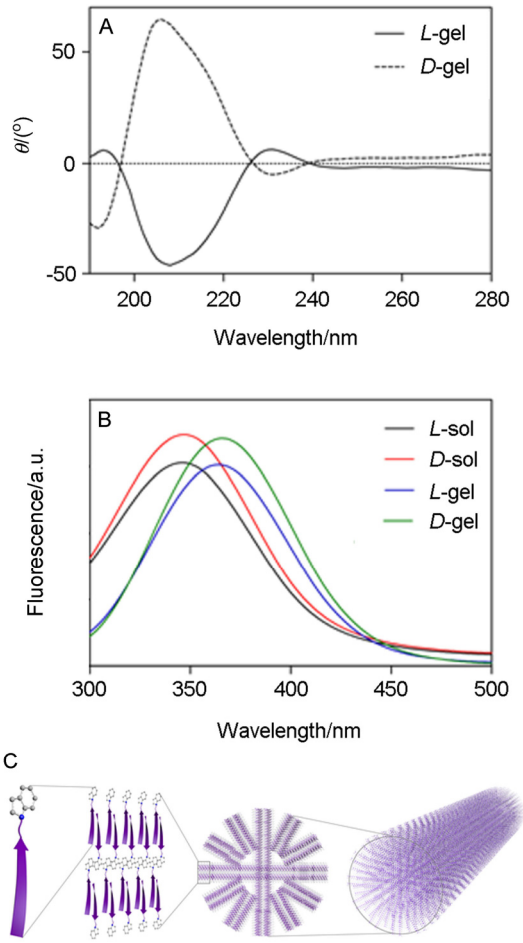


Figure 3 (A) The circular dichroism (CD) spectra of the hydrogels and (B) the emission spectra (excitation wavelength = 260 nm) of **1** and **2** in solution and in hydrogel. (C) Possible molecular arrangement of the peptide in nanofibers.

wt%), while PTZ-GFFY was a super-gelator with an MGC value of 0.01 wt%. These results were consistent with the conclusion in our previously published work that PTZ-GFFY with an additional glycine could form more extensive hydrogen bonds than peptides with capping groups such as Fmoc, Nap or Indol.[12a] We found a near mirror CD spectrum from *D*-gel, compared with that from *L*-gel, suggesting that compound **2** also adopted *β*-sheet conformation in the gel. Figure 3B showed the emission spectra of both solutions and gels. Both solutions of compounds **1** and **2** exhibited a peak centered at 349 nm. This peak showed a red-shift to about 368 nm in both gels, suggesting that the packing of indole groups was more efficient in gel stage than that in solutions. Based on these information, we proposed a possible molecular arrangement of the peptide in nanofibers (Figure 3C). Assisted by the π-π interactions between aromatic capping group of indole and hydrogen bond interactions between peptides (shown as purple ribbons with arrows), compound **1** or **2** adopted an anti-parallel packing mode with each other, and therefore helped to extend the supramolecular chain to form nanofibers.

We then tested the potency of both gels as vaccine adjuvants. The protein ovalbumin (OVA) was chosen as a model antigen to be physically mixed with the gel at a weight ratio of (peptide∶OVA＝10∶1). Four groups of C57BL/6J mice were injected with OVA, aluminium hydroxide containing OVA (Alum∶OVA＝25∶1), *L*-gel containing OVA (*L*-gel), *D*-gel containing OVA (*D*-gel), respectively at day 0. A secondary immunization was performed at day 14. The bleed of all mice were collected at day 21 and an ELISA assay was performed to determine the anti-OVA IgG titer. As shown in Figure 4A, soluble OVA group exhibited poor immune responses, while both hydrogel groups showed significantly higher anti-OVA IgG antibody productions. *D*-gel group was more potent to increase IgG production than *L*-gel group, which was similar to our recent results that the other two *D*-peptide-based hydrogels could increase anti-OVA titer more significantly than their *L*-counterparts.[5b,5c] For IgG isotopes, both gels could significantly increase the production of IgG1, IgG2a, and IgG2b than free OVA, and *D*-gel was more potent than *L*-gel (Figures 4B, 4C and 4D). We believe the reason for *D*-gel showing more efficacy than *L*-gel is the same with that proposed in our recently published work, that the release of OVA from *D*-gel was faster than that from *L*-gel in lysosome because the binding between protein OVA and *L*-nanofiber was stronger than that between OVA and *D*-nanofiber, resulting in more antigen escape from lysosome and release into cytosol, which were important for stronger immune responses.

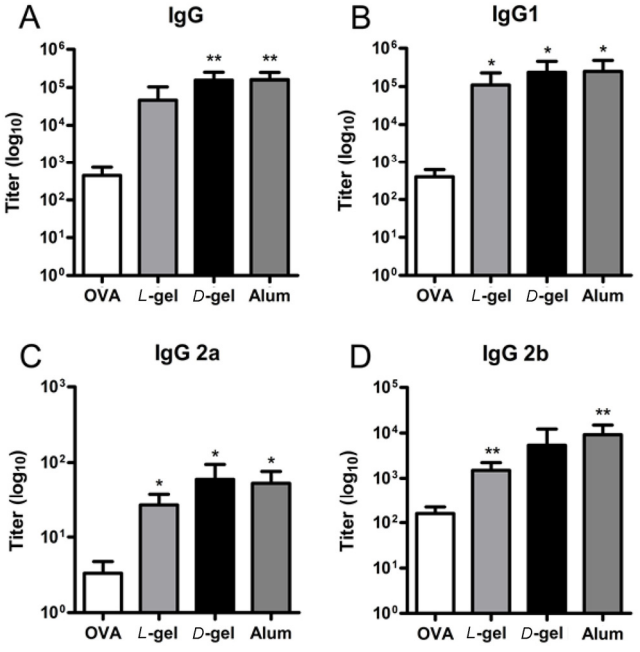


Figure 4 The production of anti-OVA IgG antibodies and its isotopes in plasma on day 21 determined by ELISA (*: $p<0.05$, **: $p<0.01$).

Our *D*-gel showed a comparable potency to evoke IgG antibody and its isotope productions with FDA approved Alum adjuvant. Even though both gels promoted the production of IFN-γ and IL-5 more than free OVA, cytokines did not reach the desired levels like Alum (Figure 5). These observations suggested that our hydrogels

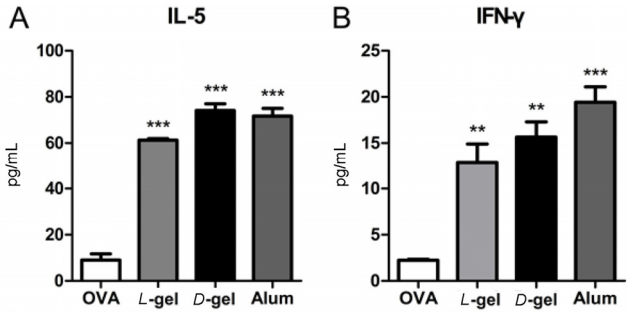


Figure 5 The production of IFN-γ and IL-5 in splenocytes culture supernatants (**: $p<0.01$, ***: $p<0.001$).

might be used as vaccine adjuvants to raise humoral immune response, which was similar to Alum adjuvant.

Moreover, we also evaluated the cytotoxicity of both gels against immune cells of RAW 264.7 cells by an MTT assay (Figure 6), and demonstrated that both gels have high biocompatibility, which confers them great potential in applications of biomedicine.

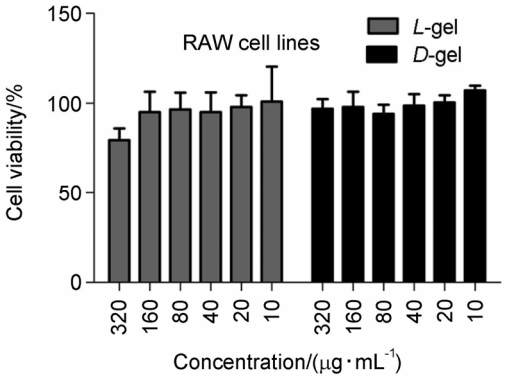


Figure 6 Cytotoxicity assay of *L*-gel and *D*-gel.

Conclusions

Our study provides a novel candidate of aromatic capping group (Indole) to construct short peptide-based hydrogelators. Our hydrogels, especially the *D*-peptide gel shares a similar potency with Alum as a vaccine adjuvant to raise humoral immune response. The good biocompatibility, ease of synthesis, and ease of vaccine formulation suggest its good promise for vaccine production. One shortcoming of the gels in this study is their poor potency to raise cellular immune response, which is believed to be very important for the development of vaccines to protect against or treat cancers, HIV, and malaria. We will opt to develop more gels and test their potency to raise both humoral and cellular immune responses.

Acknowledgement

This work is supported by National Natural Science Foundation of China (31370964, 51403105), Science and Technology Guiding Project of Guangdong Province (2013B091500071), and Program for Changjiang Scholars and Innovative Research Team in University (IRT13023).

References

[1] Heath, W. R.; Carbone, F. R. *Nat. Rev. Immunol.* **2001**, *1*, 126.

[2] (a) Bachmann, M. F.; Jennings, G. T. *Nat. Rev. Immunol.* **2010**, *10*, 787; (b) Goldberg, M. S. *Cell* **2015**, *161*, 201; (c) Kim, J.; Li, W. A.; Choi, Y.; Lewin, S. A.; Verbeke, C. S.; Dranoff, G.; Mooney, D. J. *Nat. Biotechnol.* **2015**, *33*, 64; (d) Moon, J. J.; Suh, H.; Bershteyn, A.; Stephan, M. T.; Liu, H.; Huang, B.; Sohail, M.; Luo, S.; Um, S. H.; Khant, H. *Nat. Mater.* **2011**, *10*, 243.

[3] (a) Hudalla, G. A.; Sun, T.; Gasiorowski, J. Z.; Han, H.; Tian, Y. F.; Chong, A. S.; Collier, J. H. *Nat. Mater.* **2014**, *13*, 829; (b) Rudra, J. S.; Tian, Y. F.; Jung, J. P.; Collier, J. H. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 622.

[4] Rudra, J. S.; Sun, T.; Bird, K. C.; Daniels, M. D.; Gasiorowski, J. Z.; Chong, A. S.; Collier, J. H. *ACS Nano* **2012**, *6*, 1557.

[5] (a) Tian, Y.; Wang, H.; Liu, Y.; Mao, L.; Chen, W.; Zhu, Z.; Liu, W.; Zheng, W.; Zhao, Y.; Kong, D. *Nano Lett.* **2014**, *14*, 1439; (b) Wang, H.; Luo, Z.; Wang, Y.; He, T.; Yang, C.; Ren, C.; Ma, L.; Gong, C.; Li, X.; Yang, Z. *Adv. Funct. Mater.* **2016**, *26*, 1822; (c) Luo, Z.; Wu, Q.; Yang, C.; Wang, H.; He, T.; Wang, Y.; Wang, Z.; Chen, H.; Li, X.; Gong, C.; Yang, Z. *Adv. Mater.* **2017**, *29*, 1601776.

[6] (a) Chen, S.; Rong, L.; Lei, Q.; Cao, P.-X.; Qin, S.-Y.; Zheng, D.-W.; Jia, H.-Z.; Zhu, J.-Y.; Cheng, S.-X.; Zhuo, R.-X.; Zhang, X.-Z. *Biomaterials* **2016**, *77*, 149; (b) Liu, J.; Liu, J.; Chu, L.; Zhang, Y.; Xu, H.; Kong, D.; Yang, Z.; Yang, C.; Ding, D. *ACS Appl. Mater. Interfaces* **2014**, *6*, 5558; (c) Su, T.; Tang, Z.; He, H. J.; Li, W. J.; Wang, X.; Liao, C. N.; Sun, Y.; Wang, Q. G. *Chem. Sci.* **2014**, *5*, 4204; (d) Das, D.; Kar, T.; Das, P. K. *Soft Matter* **2012**, *8*, 2348; (e) Ischakov, R.; Adler-Abramovich, L.; Buzhansky, L.; Shekhter, T.; Gazit, E. *Bioorg. Med. Chem.* **2013**, *21*, 3517; (f) Rad-Malekshahi, M.; Lempsink, L.; Amidi, M.; Hennink, W. E.; Mastrobattista, E. *Bioconjug. Chem.* **2016**, *27*, 3.

[7] (a) Pires, R. A.; Abul-Haija, Y. M.; Costa, D. S.; Novoa-Carballal, R.; Reis, R. L.; Ulijn, R. V.; Pashkuleva, I. *J. Am. Chem. Soc.* **2015**, *137*, 576; (b) Kuang, Y.; Shi, J.; Li, J.; Yuan, D.; Alberti, K. A.; Xu, Q.; Xu, B. *Angew. Chem., Int. Ed.* **2014**, *53*, 8104; (c) Li, J.; Kuang, Y.; Shi, J.; Zhou, J.; Medina, J. E.; Zhou, R.; Yuan, D.; Yang, C.; Wang, H.; Yang, Z.; Liu, J.; Dinulescu, D. M.; Xu, B. *Angew. Chem., Int. Ed.* **2015**, *54*, 13307; (d) Alam, S.; Panda, J. J.; Mukherjee, T. K.; Chauhan, V. S. *J. Nanobiotechnol.* **2016**, *14*, 26.

[8] (a) Ren, C.; Wang, H.; Mao, D.; Zhang, X.; Fengzhao, Q.; Shi, Y.; Ding, D.; Kong, D.; Wang, L.; Yang, Z. *Angew. Chem., Int. Ed.* **2015**, *54*, 4823; (b) Ren, C.; Zhang, J.; Chen, M.; Yang, Z. *Chem. Soc. Rev.* **2014**, *43*, 7257; (c) Peltier, R.; Chen, G.; Lei, H.; Zhang, M.; Gao, L.; Lee, S. S.; Wang, Z.; Sun, H. *Chem. Commun.* **2015**, *51*, 17273; (d) Du, X.; Zhou, J.; Shi, J.; Xu, B. *Chem. Rev.* **2015**, *115*, 13165.

[9] (a) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. *Chem. Rev.* **2014**, *114*, 1973; (b) Raeburn, J.; Cardoso, A. Z.; Adams, D. J. *Chem. Soc. Rev.* **2013**, *42*, 5143; (c) Morris, K. L.; Chen, L.; Raeburn, J.; Sellick, O. R.; Cotanda, P.; Paul, A.; Griffiths, P. C.; King, S. M.; O'Reilly, R. K.; Serpell, L. C.; Adams, D. J. *Nat. Commun.* **2013**, *4*, 1480; (d) Sun, Z.; Li, Z.; He, Y.; Shen, R.; Deng, L.; Yang, M.; Liang, Y.; Zhang, Y. *J. Am. Chem. Soc.* **2013**, *135*, 13379; (e) He, M.; Li, J.; Tan, S.; Wang, R.; Zhang, Y. *J. Am. Chem. Soc.* **2013**, *135*, 18718; (f) Zhou, J.; Du, X.; Li, J.; Yamagata, N.; Xu, B. *J. Am. Chem. Soc.* **2015**, *137*, 10040; (g) Nanda, J.; Biswas, A.; Adhikari, B.; Banerjee, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 5041; (h) Das, A. K.; Maity, I.; Parmar, H. S.; McDonald, T. O.; Konda, M. *Biomacromolecules* **2015**, *16*, 1157; (i) Piepenbrock, M.-O. M.; Lloyd, G. O.; Clarke, N.; Steed, J. W. *Chem. Rev.* **2010**, *110*, 1960; (j) Boekhoven, J.; Hendriksen, W. E.; Koper, G. J. M.; Eelkema, R.; van Esch, J. H. *Science* **2015**, *349*, 1075; (k) Bowerman, C. J.; Nilsson, B. L. *J. Am. Chem. Soc.* **2010**, *132*, 9526; (l) Martin, A. D.; Robinson, A. B.; Mason, A. F.; Wojciechowski, J. P.; Thordarson, P. *Chem. Commun.* **2014**, *50*, 15541.

[10] Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

[11] (a) Kuang, Y.; Xu, B. *Angew. Chem., Int. Ed.* **2013**, *52*, 6944; (b) Kuang, Y.; Du, X.; Zhou, J.; Xu, B. *Adv. Healthcare Mater.* **2014**, *3*, 1217; (c) Zheng, W.; Gao, J.; Song, L.; Chen, C.; Guan, D.; Wang, Z.; Li, Z.; Kong, D.; Yang, Z. *J. Am. Chem. Soc.* **2013**, *135*, 266; (d) Li, J.; Gao, Y.; Kuang, Y.; Shi, J.; Du, X.; Zhou, J.; Wang, H.; Yang, Z.; Xu, B. *J. Am. Chem. Soc.* **2013**, *135*, 9907; (e) Miao, X.; Cao, W.; Zheng, W.; Wang, J.; Zhang, X.; Gao, J.; Yang, C.; Kong, D.; Xu, H.; Wang, L.; Yang, Z. *Angew. Chem., Int. Ed.* **2013**, *52*, 7781; (f) Wang, H.; Wang, Y.; Zhang, X.; Hu, Y.; Yi, X.; Ma, L.; Zhou, H.; Long, J.; Liu, Q.; Yang, Z. *Chem. Commun.* **2015**, *51*, 14239.

[12] (a) Ou, C.; Zhang, J.; Zhang, X.; Yang, Z.; Chen, M. *Chem. Commun.* **2013**, *49*, 1853; (b) Li, D.; Shi, Y.; Wang, L. *Chin. J. Chem.* **2014**, *32*, 123.

[13] Greenfield, N. J. *Nat. Protoc.* **2006**, *1*, 2876.

(Pan, B.; Fan, Y.)

