Mesoporous Materials Modified by Aptamers and Hydrophobic Groups Assist Ultra-sensitive Insulin Detection in Serum

Chang Lei,[a] Chun Xu,[a] Owen Noonan,[a] Anand Kumar Meka,[a] Long Zhang,[a] Amanda Nouwens[a,b] and Chengzhong Yu*[a]

[a]Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, QLD 4072, Australia

[b]School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD 4072, Australia

Supporting information

1. Experimental section

1.1 Chemicals

Insulin (MW 5807.6), Tetraethylorthosilicate (TEOS), Tetramethoxyorthosilicate (TMOS), trimethylchlorosilane (TMCS), $EO_{20}PO_{70}EO_{20}$ [donated as P123, where EO is poly-(ethylene oxide) and PO is poly (propylene oxide)], α-cyano-4-hydroxycinnamic acid (CHCA, 99%), 3-Aminopropyl(diethoxy)methylsilane(APDEMS), 3-aminopropyltriethoxysilane (APTES), horse serum, sodium sulfate (Na_2SO_4), 2-(N-morpholino)ethanesulfonic acid (MES, pH 5.5), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Hydrochloric acid (HCl) and ethanol were received from ChemSupply. Toluene, Acetonitrile (ACN, 99.9%) and trifluoroacetic acid (TFA, 99.8%) were purchased from Merck. COOH-IBA (COOH-ACAGGGGTGTGGGGACAGGGGTGTGGGG) was ordered from Thermo Fisher Scientific. Human insulin ELISA kit was purchased from Life Technologies (Australia Pty Ltd). All reagents were used as received without further purification. Deionized water (DI water) (18.2 mV cm) used for all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA).

1.2 Materials Synthesis

Silica materials (denoted SBA-15 and MOSF) were synthesized using modified literature methods, and the experimental processes are given as follows:

For SBA-15, P123 (1.0g) was dissolved in 2M HCl (30g) solution at 38 °C. Then TEOS (2.08g) was added into the homogeneous solution under stirring at 38 °C for 24h. The resulting white precipitate was filtered and mixed with 40g of deionized water and the pH was adjusted to 1.65 using 1 M HCl. The reaction mixture was heated at 130 °C for another 24h. The as-synthesized SBA-15 was filtered and dried at room temperature. Calcination was performed in furnace at 550 °C for 5h to completely eliminate the surfactants.[1, 2]

For MOSF, it includes a synthesis of MOSF and a further modification of $–CH_3$. For MOSF, At 35 °C, 1.0 g of P123 and 1.7 g of Na_2SO_4 (0.4 M) were dissolved in 30 g of pH=5.0 NaAc-HAc buffer solution (Ct=0.02 M, where Ct=CNaAc + CHAc) to form a homogenous solution under stirring. To this mixture solution, 1.52 g of TMOS was added under stirring for 5 minutes. The resultant mixture was kept at static condition for 24 h and then hydrothermally treated at 100 °C for another 24 h. The product was filtered and washed repeatedly with water to remove inorganic salts, and then dried at room temperature. The final MOSF products were obtained by calcination at 550 °C for 5 h.[3]

1.3 Methyl modification

1 g of silica was added into 30 ml of 5 wt % TMCS-toluene solution with stirring at 70 °C, 24h. The mixture was centrifuged and extensively washed with ethanol to rinse away any residual chemicals. Finally, the powder was dried at 50 °C.[4]

1.4 Amino modification

1 g of SBA-15 was suspended in 30 ml of 4mmol APTES-toluene solution, and the mixture was refluxed at 110 °C for 20 h. The products were centrifuged and washed with ethanol for 3 times. In the last step, the powder was dried room temperature overnight.[5]

1.5 Co-modification of IBA and CH_3

1 g of SBA-15 was dispersed in 30 ml of 4 mmol APDEMS-toluene solution. The mixture was stirring at 70 °C overnight. After washing with ethanol for 3 times, the product was dried at room temperature. SBA-15 with modifications of both CH_3 and NH_2 is denoted SBA-15-CH_3-NH_2.

For the nanoparticles with co-modification only on the outer surface [SBA-15-CH_3-IBA (out)]: the as-synthesized SBA-15 (with surfactant) was applied to the reaction with APDEMS directly. With the existence of surfactant, the CH_3 and NH_2 were only modified on the outer surface of SBA-15. Then, the surfactant of modified nanoparticles was removed by extraction with methanol-HCl (128 ml methanol and 8ml HCl) under stirring for 48 h. The product was dried at room temperature and ready for the conjugation with IBA.

For the conjugation of IBA: 100 mg of SBA-15-CH_3-NH_2 was suspended in 25 ml MES buffer and sonicated for a few minute to have a well-dispersed and homogenous solution. To this solution, EDC (60 mg dissolved in 25 ml of MES buffer) and NHS (160 mg dissolved in 25 ml of MES buffer) were added, followed by 25 nmol of COOH-IBA (100μM in DNA-free H_2O). The reaction was conducted at room temperature with gentle shaking for 3 h. After 3 times washing with phosphate buffered saline buffer (PBS buffer, pH7.2), the SBA-15-CH_3-IBA conjugates were stored at 4°C and ready for use.

1.6 Materials characterization

Small-angle X-ray scattering (SAXS) was performed using an Anton-Parr SAXSess small-angle X-ray scattering system (Cu KR radiation, 35kV, 30 mA). The samples were placed onto the transparent tape as a thin layer and mounted onto the sample holder, analysed for the scattering peaks, and for background/blank only tape is mounted onto the sample holder. All the samples were analysed using following parameters: Number of exposure to average per experiment (scan rate) = 1000; Exposure time=0.2 seconds; Width of detection area= 30 mm; Wavelength =0.1542 nm; Sample detector distance = 309 nm.

Nitrogen-sorption isotherms of the samples were obtained by a Micromeritics Tristar II 3020 system at 77 K. Before the measurements, about 50 mg of each sample was weighted and degassed at 100 °C for at least 8 h in vacuum. After degassing, the samples were weighted again to get the accurate weight and then transferred to the Tristar system for testing.

Fourier transform infrared (FTIR) spectra were collected with ThermoNicolet Nexus 6700 FTIR spectrometer equipped with Diamond ATR (attenuated total reflection) Crystal. The platform and diamond were cleaned with ethanol before placing each sample. For each spectrum, 128 scans were collected at a resolution of 4 cm^{-1} over the range 500–4000cm^{-1}.

Transmission electron microscopy (TEM) images were directly taken with a JEOL 1010 microscope operated at 100 kV. Samples were dispersing in ethanol before coating on Cu grids covered with carbon films. After evaporating the ethanol, the Cu grids were ready for TEM testing.

ζ potential measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments. The samples were dispersed
in Milli-Q water before testing.

X-ray photoelectron spectroscopy (XPS) measurements were performed with a Kratos Axis Ultra X-ray photoelectron spectrometer (Perkin-Elmer). All spectra were acquired at a basic pressure of 2×10^{-7} Torr with Mg Kα excitation at 15 kV. All the results were analysed using the CasaXPS software and corrected by referencing the C 1s peak at 284.8 eV.

1.7 Enrichment of insulin in serum

The insulin serum solution (10% horse serum) was prepared freshly every time before experimentation with a stepwise dilution method. Firstly, 5 μl (10 μg/ μl) MOSF-CH_3 was added into the insulin serum sample (500 μl) for the first step purification. After 5 min of stirring and 10 min of centrifugation, the supernatant was transferred to another Eppendorf tube for enrichment, and the sediment was spotted onto the MALDI MPT 384 plate and mixed with 1 μl CHCA solution before test. In the next step, 1 μl (10 μg/ μl) SBA-15-CH_3-IBA was added into the purified solution for further enrichment. Stirring (5 min) and centrifugation (10 min) were conducted before removing the supernatant, sediment was finally mixed with CHCA for MS analysis.

All the samples were analyzed on a Bruker Autoflex TOF/TOF III Smart beam. The mass spectra were obtained in the LP-PepMix mode via an accumulation of 200 laser shots at 10 different sites under a laser intensity of 39% for data collection and calibrated. Three standard peptides, Angiotensin II (Mw = 1046.5 Da), ACTH-Clip (Mw = 2465.2 Da) and Somatostatin 28 (Mw 3147.5 Da) were used for calibration purposes to reduce variability.

1.8 Pre-enrichment enhanced ELISA

The standard insulin solutions in a low concentration range (0.005 ~ 0.00025 ng ml^{-1}) were prepared freshly by stepwise diluting the standard solution #1 in the ELISA kit (containing 0.2 ng ml^{-1} of insulin in human serum). For these intact insulin solutions, 50 μl of each was kept as the control group. For the insulin pre-enrichment, 30 μl (10 mg/ml) of MOSF-CH_3 was added into each sample (5 ml) for the first step purification. After stirring and centrifugation, the supernatant was separated and transferred for the second enrichment step. Then, 10 μl (10 mg/ml) SBA-15-CH_3-IBA was added to enrich insulin from the purified supernatant with 5 mins mixing. After centrifugation, the supernatant was removed and 2 ml of 80% ACN(0.1%TFA) was added to the sediment for elution. In the next step, the eluted solution was centrifuged and the supernatant with insulin was transfered to another tube for vacuum drying at 45°C overnight. The sample was ready for ELISA test after redissolving with 50 μl DI water.

ELISA test was conducted following the instruction in the commercial kit, the mechamism is showing in Figure S6. Typically, 50 μl of each standards/control/samples was added into the wells together with 50 μl anti-insulin HRP, incubating for 30 min at room temperature. The liquid was decanted thoroughly and the walls were washed 3 times with the diluted wash solution before adding 100 μl stabilized chromogen. After 15 min, 100 μl of stop solution was added and the plate was measured by reading the absorbance at 450 nm using a Synergy HT microplate reader within 1 h. All the standards/control/samples were run in duplicate.

To test the recovery rate, a fix amount of standard (0.005 ng) was spiked to sample 1 with 0.01 ng insulin, and the amount of spikd standard plus sample 1 is sample 2. Then, both sample 1 and 2 were pre-treated by the designed combo-pore-CH_3-IBA approach following with ELISA test, and the results were calculated according to the low concentration standard curve (Figure 3b). Theoratically, the difference of insulin amount between sample 1 and 2 is the spiked amount (0.005 ng). Thus, the accurancy of our combo-pore-CH_3-IBA enhanced ELISA is represented by the ratio (recovery rate) of testing spiked standard to 0.005 ng (theoratical spiked amount).

2. Table and Figures

Table S1. Methods and LOD of insulin detection in references

Method	Quantitative/Qualitative	LOD (ng ml^{-1})	Ref.no
RIAs	Quantitative	10.4 (Cerebrospinal fluid)	[6]
Human Insulin-Enzyme Amplified Sensitivity Immunoassay (EASIA)	Quantitative	18 (Urine)	[7]
SPE-Electrospray LC-MS with bovine insulin as internal standard	Quantitative	1 (Blood)	[8]
SPE-IAC-LC-MS/MS	Quantitative	290 (Urine)	[9]
Micro-SPE with molecularly imprinted polymer-sensor	Quantitative	0.009 (Serum)	[10]
Molecularly imprinted SPE cartridge with HPLC	Quantitative	0.7 (Plasma); 0.1 (Urine)	[11]
Immunoaffinity-Nano-LC-MS	Quantitative	2.9 (Urine)	[12]
Immunoaffinity-LC/MS/MS	Quantitative Qualitative	0.05 (Equine urine) 0.5 (Plasma)	[13] [14]
Aptamer-MALDI-TOF MS	Qualitative	20 (Serum)	[15]
Photoluminescence (PL) emission of aptamer-coated SWNTs	Quantitative	58 (Cell matrix)	[16]
HPLC with an UV detector	Quantitative	2.27 (Plasma)	[17]
HPLC-UV	Quantitative	350 (Rat Plasma)	[18]

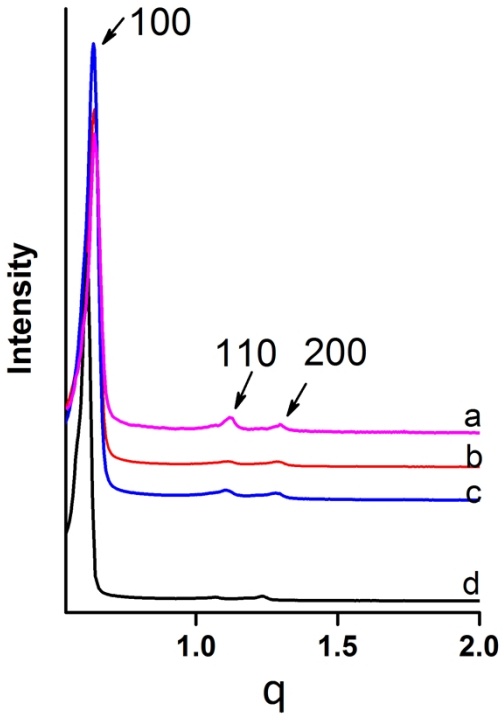


Fig. S1 SAXS patterns of a) SBA-15; b) SBA-15-NH_2; c) SBA-15-CH_3-NH_2 and d) SBA-15-CH_3-NH_2 (out).

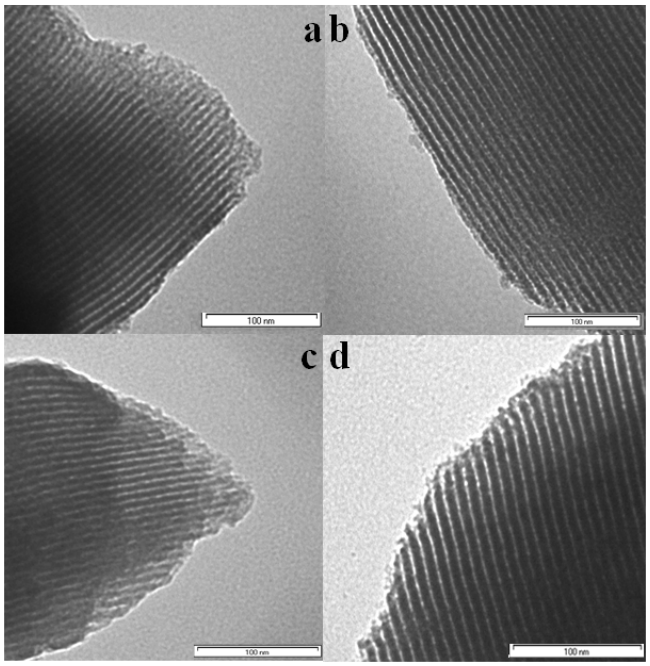


Fig. S2 TEM images of (a) SBA-15, (b) SBA-15-CH_3-NH_2, c) SBA-15-NH_2 and (d) SBA-15-CH_3-NH_2 (out).

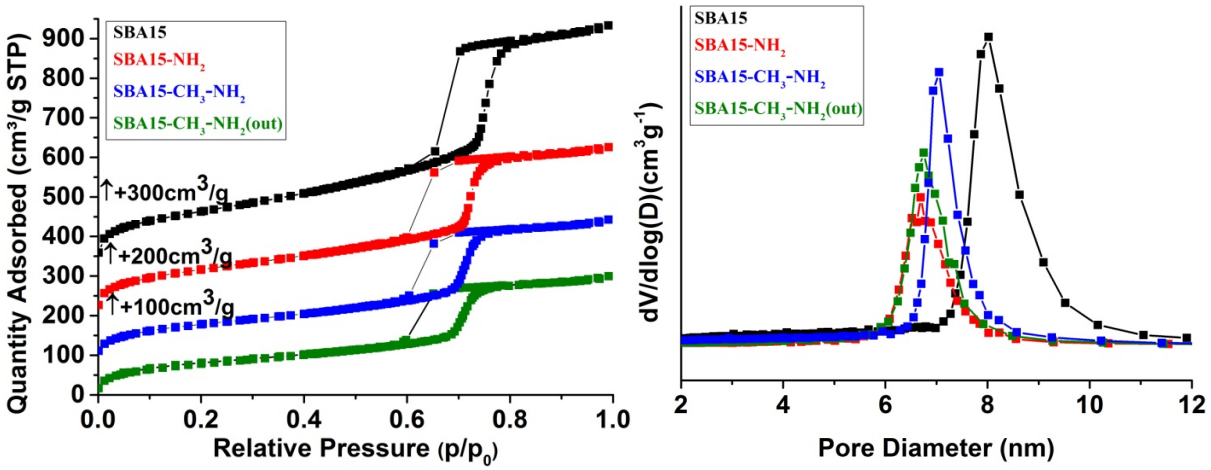


Fig. S3 Nitrogen adsorption-desorption isotherms and pore size distribution curves of SBA-15 with& without modifications.

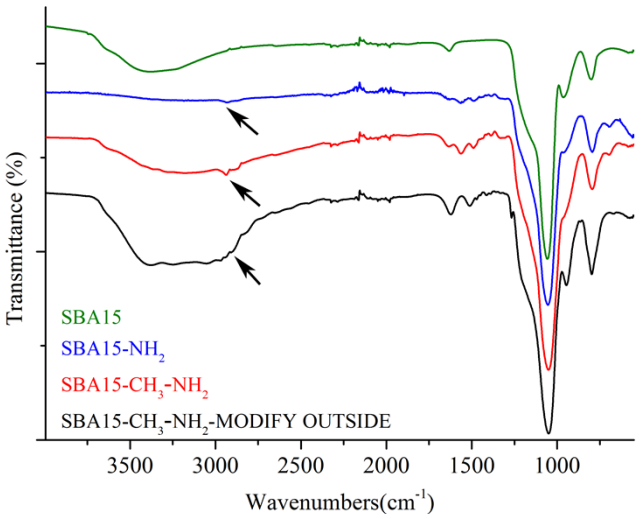


Fig. S4 FTIR spectra of SBA-15, SBA-15-NH_2, SBA-15-CH_3-NH_2 and SBA-15-CH_3-NH_2 (out). Black arrows show the peaks of -CH_3 group.

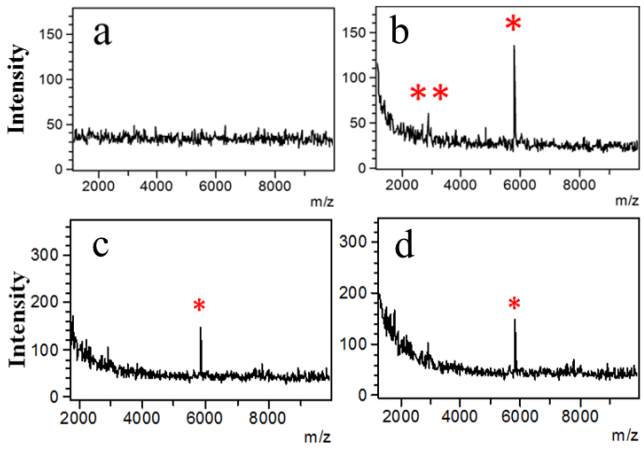
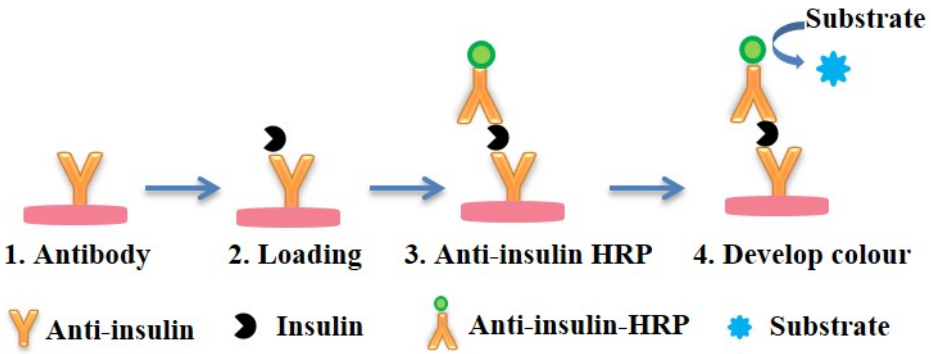


Fig. S5 MS spectra obtained from a)10ng ml^{-1} human insulin (in horse serum); b) 0.05 ng ml^{-1} human insulin (in horse serum) with pre-treatment by designed SBA15-CH_3-IBA; c)1ng ml^{-1} human insulin (in horse serum) with pre-treatment by SBA15-IBA; d) 0.5 ng ml^{-1} human insulin in horse serum with pre-treatment by SBA15-CH_3-IBA (out); *stands for the single charged molecular ion peak, ** stands for the double charged molecular ion peak of human insulin molecule.



Scheme S1. Illustration showing the mechanism of sandwich-like ELISA.

Reference

1. S. Jarnbhrunkar, M. H. Yu, J. Yang, J. Zhang, A. Shrotri, L. Endo-Munoz, J. Moreau, G. Q. Lu and C. Z. Yu, *J Am Chem Soc*, 2013, **135**, 8444-8447.
2. D. H. Pan, P. Yuan, L. Z. Zhao, N. A. Liu, L. Zhou, G. F. Wei, J. Zhang, Y. C. Ling, Y. Fan, B. Y. Wei, H. Y. Liu, C. Z. Yu and X. J. Bao, *Chem Mater*, 2009, **21**, 5413-5425.
3. H. N. Wang, X. F. Zhou, M. H. Yu, Y. H. Wang, L. Han, J. Zhang, P. Yuan, G. Auchterlonie, J. Zou and C. Z. Yu, *J Am Chem Soc*, 2006, **128**, 15992-15993.
4. X. S. Zhao and G. Q. Lu, *J Phys Chem B*, 1998, **102**, 1556-1561.
5. C. Xu, Y. T. Niu, A. Popat, S. Jambhrunkar, S. Karmakar and C. Z. Yu, *J Mater Chem B*, 2014, **2**, 253-256.
6. W. Kern, C. Benedict, B. Schultes, F. Plohr, A. Moser, J. Born, H. L. Fehm and M. Hallschmid, *Diabetologia*, 2006, **49**, 2790-2792.
7. S. Pichini, R. Ventura, I. Palmi, S. di Carlo, A. Bacosi, K. Langohr, R. Abellan, J. A. Pascual, R. Pacifici, J. Segura and P. Zuccaro, *J Pharmaceut Biomed*, 2010, **53**, 1003-1010.
8. S. M. Darby, M. L. Miller, R. O. Allen and M. LeBeau, *J Anal Toxicol*, 2001, **25**, 8-14.
9. T. Kuuranne, A. Thomas, A. Leinonen, P. Delahaut, A. Bosseloir, W. Schanzer and M. Thevis, *Rapid Commun. Mass Spectrom.*, 2008, **22**, 355-362.
10. M. P. Tiwari and B. B. Prasad, *J Chromatogr A*, 2014, **1337**, 22-31.
11. M. M. Moein, M. Javanbakht and B. Akbari-adergani, *Talanta*, 2014, **121**, 30-36.
12. A. Thomas, W. Schanzer, P. Delahaut and M. Thevis, *Drug Test Anal*, 2009, **1**, 219-227.
13. E. N. M. Ho, T. S. M. Wan, A. S. Y. Wong, K. K. H. Lam and B. D. Stewart, *J Chromatogr A*, 2011, **1218**, 1139-1146.
14. M. Thevis, A. Thomas, P. Delahaut, A. Bosseloir and W. Schanzer, *Anal Chem*, 2005, **77**, 3579-3585.
15. X. Y. Zhang, S. C. Zhu, C. H. Deng and X. M. Zhang, *Chem Commun*, 2012, **48**, 2689-2691.
16. T. G. Cha, B. A. Baker, M. D. Sauffer, J. Salgado, D. Jaroch, J. L. Rickus, D. M. Porterfield and J. H. Choi, *Acs Nano*, 2011, **5**, 4236-4244.
17. G. Khaksa, K. Nalini, M. Bhat and N. Udupa, *Anal Biochem*, 1998, **260**, 92-95.
18. S. Ravi, K. K. Peh, Y. Darwis, B. K. Murthy and T. R. R. Singh, *Chromatographia*, 2007, **66**, 805-809.