Supporting information

Local release of highly loaded antibodies from functionalized nanoporous support for cancer immunotherapy

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Experimental section

FMS and FMS-antibody. Hexagonally ordered mesoporous silica (SBA-15) of pore size 300 Å and surface area of 533 m^2/g were prepared according to procedures modified from our earlier work.[1,2] In a typical preparation of mesoporous silica with 300 Å pores, 12.0 g of Pluronic P-123 (MW=5,800) was dissolved in 2 M HCl solution (360 mL) at 40^{o}C. Then 18.0 g of mesitylene and 25.5 g of tetraethylorthosilicate (TEOS) were added to the milky solution and stirred for 18 h at the same temperature. The mixture was transferred into a Teflon-lined autoclave and heated up to 100^{o}C for 24 h without stirring. The white precipitate was collected by filtration, dried in air, and finally calcined at 550^{o}C for 6 hours. A controlled hydration and condensation reaction was used to introduce functional groups into unfunctionalized mesoporous silica (UMS).[1,2] A coverage of 2% (or 20%) HOOC-FMS, HO_3S-FMS, HS-FMS or NH_2-FMS means 2% (or 20%) of the total available silanol groups (5 x 10^{18} silanol groups per square meter[1,2]) of UMS would be silanized with the trimethoxysilane with the functional group HOOC, HO_3S, HS or NH_2.

In a typical procedure of 2% HOOC-FMS synthesis (300 Å pores), 1.0 g of mesoporous silica was first suspended in toluene (60 mL) and pretreated with water (0.32 mL) in a three-necked 250 mL round-bottom flask, which was fitted with a stopper and reflux condenser. This suspension was stirred vigorously for 2 h to distribute the water throughout the mesoporous matrix, during which time it became thick and homogeneous slurry. At this point, 15.5 mg of tris-(methoxy)cyanoethylsilane (TMCES, MW = 175.26) was added and the mixture was refluxed for 6 h. The mixture was allowed to cool to room temperature and the product was collected by vacuum filtration. The treated mesoporous silica was washed with ethyl alcohol repeatedly and dried under vacuum. To hydrolyze cyano groups (CN- would be hydrolyzed into HOOC- as the functional group), 10 mL of 50% of H_2SO_4 solution was added to the mixture and refluxed for 3 h. The

product was filtered off and washed with water extensively. Other samples were synthesized by the same procedure except different amounts of organosilanes were added based on their surface areas, and no hydrolysis step when functionalizing with tris-(methoxy)aminopropylsilane (TMAPS, NH_2- as the functional group) and tris-(methoxy)mercaptopropylsilane (TMMPS, HS- as the functional group). HO_3S-FMS was prepared via oxidation of HS-FMS by 30% (w/w) H_2O_2.

Typically, an aliquot of 2.0–8.0 mg of FMS was added in a 1.8-mL tube for incubation with 200–1600 μL of the antibody stock. Based on the preliminary experiments, at least 0.5–1.0 mg antibody was used for incubation with per mg of FMS so that FMS was loaded to saturation with the antibody. The incubation was carried out at 18-21^{o}C shaking at 1400 min^{-1} on an Eppendorf Thermomixer 5436 for 12–24 h. The antibody stock in the absence of FMS was also shaken under the same conditions for comparison. Then the FMS-antibody composites were separated by centrifugation and removing the supernatant (the elution number: 0). The amounts of proteins were measured by Bradford method using bovine gamma globulin as standards. To test the in vitro gradual release of IgGs from FMS, the IgGs were loaded to saturation in 1.0 mg of FMS. Then, 250 μl of the fresh simulated body fluid buffer or pH 7.4, PBS was used for each subsequent elution by incubating and shaking FMS-IgG in the elution buffer for 5-10 minutes. The amounts of the subsequently released IgGs were measured at 210 nm using IgGs as standards.

High resolution TEM was carried out on a Jeol JEM 2010 Microscope with a specified point-to-point resolution of 0.194 nm. The operating voltage on the microscope was 200 keV. Fluorescence emission spectra were measured with a Fluoro Max-2 fluorometer (SPEX, Edison, NJ).

Mice and tumor cells. Six- to eight-week-old female C3H/HeN mice were purchased (Charles River Laboratories, Wilmington, MA). The SW1C clone of the K1735 melanoma is of C3H/HeN origin.[3] The animal facilities are ALAC certified, and our protocols are approved by University of Washington's IACUC Committee.

In vivo antibody release assay. 6-8 week female C3H mice were transplanted s.c. on one side of the back with 10^6 SW1-WT tumor cells. When the tumor size reached 3 mm by 3 mm, 0.885 mg of 20% HOOC-FMS Rat IgG-FITC, containing 0.1 mg Rat IgG-FITC, was injected into the tumor. Mice were euthanized at the indicated time point. The tumors were removed, cut into small pieces, digested in the tumor digestion medium (Hank's balanced salt solution with collagenase, hyaluronidase, and DNase) for 2 h at 37°C with shaking. The supernatant was harvested by centrifuge. The fluorescence intensity was measured at OD535 by ELISA reader.

Animal studies. Mice were transplanted s.c. on both sides of the back, with 10^6 tumor cells. When the tumors were 3-5 mm in mean diameter, mice in the experimental groups were injected s.c. with FMS containing 0.5-0.8 mg anti-CTLA4,[4] or rat IgG, while the control groups got FMS alone, PBS or the same amount of anti-CTLA4 by i.p.. Tumor growth was assessed by measuring the two largest perpendicular diameters and reported as average tumor volume (in mm^3) by the formula ($length^2 \times width/4$). Statistical analysis of these results was done by *t*-test and one-way ANOVA test. All statistical tests were two-sided.

Mouse IgG loading density in FMSs and gradual release of IgG from FMSs

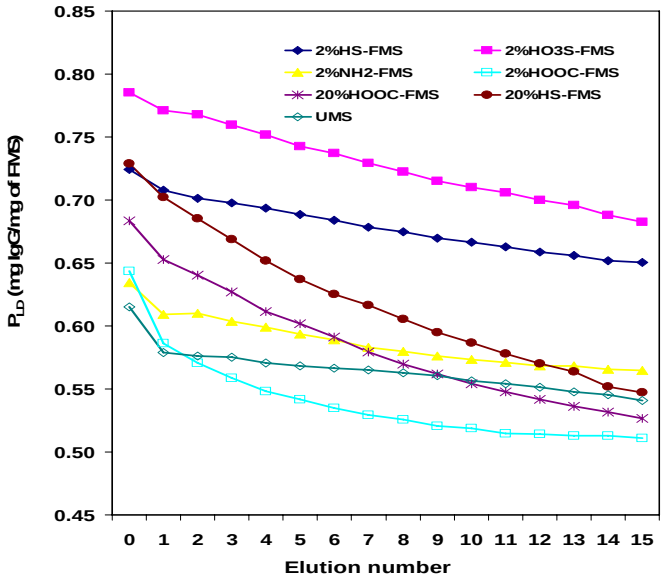

Fig. S1. Mouse IgG loading density in FMS and gradual release of IgG from FMSs. The mouse IgGs were loaded to saturation in 1.0 mg of FMS in pH 7.4, PBS. Then, the FMS-IgGs were obtained by centrifuge and removing the supernatant (the elution number: 0). Then, 250 µl of the fresh pH 7.4, PBS was used for each subsequent elution by incubating and shaking FMS-IgG in the elution buffer for 10 minutes.

Relative activity of continuously released antibody from FMS

Table S1. Relative activity of continuously released antibody from FMS*

FMSs	Relative binding activity of anti-CIP released from FMSs			
	24 h	48 h	72 h	96 h
20% HO_3S-FMS	0.76	1.26	1.14	1.14
20% HOOC-FMS	1.25	0.77	1.15	1.02
20% HS-FMS	1.18	1.32		
20% NH_2-FMS	0.82	0.94	1.09	1.10
2% HO_3S-FMS	0.93	1.00	0.78	1.18

*Sample preparation: Anti-CIP was shaken with individual FMS in pH 7.4, PBS for every 24 h, then centrifuged and the supernatant was taken out and measured. The same volume of the fresh buffer was added after taking the supernatant out each time.

To confirm that the released antibody can still maintain the binding activity to its antigen, we incubated commercially available rabbit anti-calf intestinal alkaline phosphatase (anti-CIP) with various FMS. The binding activity for antigen of the released anti-CIP from FMS was measured by surface plasma resonance to determine whether FMS binding had any deleterious effect on antibody activity. The activity was calculated assuming that if 100% active, 148 RU of the antibody would exhibit a maximum antigen binding of 116 RU, 116/148 = 88% active and assigned a relative activity ratio of 1. Thus, the relative activities of the released anti-CIP from FMS were measured (Table S1). Although there is some data variation, the released anti-CIP maintained their binding activity.

In vivo release of fluorescent dye-labeled IgG from FMS

To monitor the local release of the antibodies from FMS in mice, we injected FITC-labeled-rat IgG (IgG-FITC) and FMS-IgG-FITC into established mouse melanomas derived from subcutaneous (s.c.) injection of cells from the SW1 clone of the K1735 melanoma.[5] There were two groups of mice, in which tumors were injected with the same amount of IgG-FITC with or without entrapment in 20% HOOC-FMS particles. Tumors and sera were harvested after 2, 4, and 8 days, and tumors were digested with digestion buffer (Hank's balanced salt solution with collagenase, hyaluronidase, and DNase). The tumor lysates were cleared by centrifugation, and the supernatants were collected. The fluorescence intensity was measured in the serum and tumor supernatants. The unreleased IgG-FITCs inside FMS were not counted because that part stays with the cell pellet.

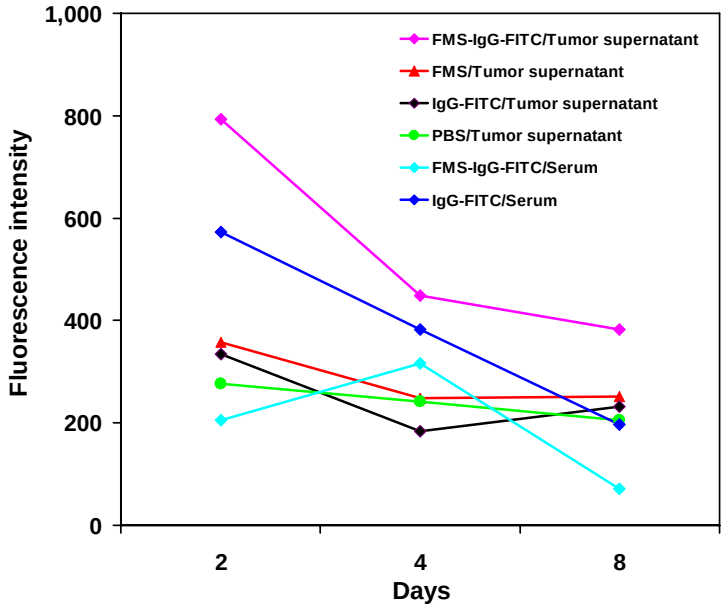

Fig. S2. Apparent distribution of FITC labeled-rat IgG in tumor and sera after injecting 0.1 mg Rat IgG-FITC free in pH 7.4, PBS or entrapped in 20% HOOC-FMS subcutaneously on one side of the mouse back. The blank pH 7.4, PBS and 20% HOOC-FMS were used as the control samples.

At the tumor site on day 2, all initially injected IgG-FITC (no FMS) was completely gone (see the control experiments, Fig. S2). In sharp contrast, for the FMS-IgG-FITC on day 2, and even on days 4 and 8, there was still significant free IgG-FITC released from the FMS particles at the tumor site. In the case when FMS-IgG-FITC was injected into the tumor, we got a higher FITC reading in tumor supernatant accompanied by a lower one in the serum (Fig. S2). We conclude that the FMS particles kept releasing the IgG-FITC, because we otherwise would not have detected any free IgG-FITC after 2, 4, and 8 days, because IgG-FITC that is not entrapped in FMS particles is distributed very quickly (Fig. S2). Interestingly, the data were the opposite when IgG-FITC (no FMS) was injected into the tumor; that is, we got a lower FITC reading in tumor supernatant accompanied by a higher one in the serum. The data clearly show that, after euthanization, FMS-IgG-FITC-injected mice had more antibodies in the whole tumor cells than did the IgG-FITC mice in the absence of FMS. These results indicate that FMS entrapping with IgG prolonged the antibody stay at the tumor site and thus facilitates sustained antibody release in tumors, offering an advantage over simply injecting antibodies into tumors.

References

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