

1 Ex Vivo Detection of Iron Oxide Magnetic Nanoparticles in Mice 2 Using Their Intrinsic Peroxidase-Mimicking Activity

3 Jie Zhuang,[†] Kelong Fan,[†] Lizeng Gao,[†] Di Lu,[†] Jing Feng,[†] Dongling Yang,[†] Ning Gu,[‡] Yu Zhang,[‡]
4 Minmin Liang,^{*†} and Xiyun Yan^{*†}

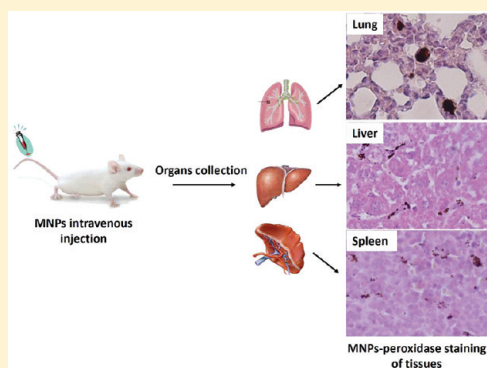
5 [†]Key Laboratory of Protein and Peptide Pharmaceutical, National Laboratory of Biomacromolecules, CAS-University of Tokyo Joint
6 Laboratory of Structural Virology and Immunology, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing
7 100101, China

8 [‡]State Key Laboratory of Bioelectronics, Southeast University, Nanjing 210096, China

9 **S** Supporting Information

10 **ABSTRACT:** Iron oxide magnetic nanoparticles (MNPs) are widely used as
11 diagnostic and therapeutic agents for biomedical applications. Quantitatively
12 analyzing biodistribution, pharmacokinetics and organ clearance of MNPs in
13 mouse models is important for understanding their in vivo behavior. In this
14 study, we developed a novel histochemical method for visualizing unlabeled
15 MNPs in mouse tissues by employing their intrinsic peroxidase-mimicking
16 activity, regarding which we reported previously that MNPs could catalyze the
17 oxidation of peroxidase substrates to produce a color reaction at the site of
18 MNPs (Gao et al. *Nanotechnol.* 2007, 2, 577–583¹). Based on this
19 MNPs-peroxidase approach, we determined the biodistribution and organ
20 clearance of MNPs by visualizing and quantifying the localization of MNPs
21 within the main organs. Compared to traditional Prussian blue assay, this
22 novel MNPs-peroxidase approach has higher sensitivity. In conclusion, the
23 developed MNPs-peroxidase approach based on intrinsic peroxidase activity of iron oxide nanoparticles was used effectively for
24 quantitative detection of MNPs in mice by histochemical staining. Presumably, other nanoparticles having intrinsic peroxidase
25 activity could also be considered.

26 **KEYWORDS:** iron oxide magnetic nanoparticle, peroxidase-mimicking activity, biodistribution, organ clearance



27 ■ INTRODUCTION

28 Iron oxide magnetic nanoparticles (MNPs) hold promise as
29 diagnostic, therapeutic and theranostic agents for a wide variety
30 of human diseases, for example as transporters for drugs or as
31 contrast agents.^{2–4} To render MNPs viable for clinical
32 translation, it is essential to understand their behavior after
33 administration into the body. However, sensitive detection of
34 MNPs in mice is difficult because nanoparticle behavior is a
35 complex functions of the surface physicochemical properties,
36 hydrodynamic diameter, solubility, stability, shape and
37 flexibility.^{5,6} Moreover, the endogenous iron in the form of
38 iron-containing proteins (e.g., hemoglobin, transferrin, and
39 ferritin) may also interfere with low tissue concentrations of
40 MNP detection. In the past several years, many optical and
41 radioisotopic indicators have been conjugated to iron oxide
42 nanoparticles as molecular imaging agents for assessing the
43 biodistribution and pharmacokinetics of MNPs in mice.^{7–9}
44 However, exogenous labeling is likely to change the surface
45 properties of nanoparticles and affect their in vivo pharmaco-
46 kinetics and tissue distribution. In addition, the unavoidable
47 detachment of labels from nanoparticles in serum often causes
48 false detection results, especially when MNPs need long-term
49 monitoring because of their slow clearance from the body.^{10,11}

Therefore, developing a method by employing their intrinsic
properties to achieve nanoparticle detection in mice will be
more advantageous. The ex vivo Prussian blue histological
staining is a convenient assay to detect the presence of MNPs
in mouse organs by using their intrinsic chemical properties.^{12–14} However, Prussian blue staining is not sensitive
enough to detect low amounts of iron in tissues. In addition,
the staining results are usually affected by endogenous ferric
iron in tissues.¹⁵

In previous studies, we first reported that ferrimagnetic
nanoparticles exhibit peroxidase activity that can catalyze the
oxidation of peroxidase substrates in the presence of H₂O₂ to
produce a color reaction, similar to that of natural peroxidases.¹
Since reported in 2007, this finding has been widely applied in
medicine, biotechnology and environmental chemistry bio-
technology.^{16–21} In this study, we developed a MNPs-
peroxidase method based on the intrinsic biochemical property

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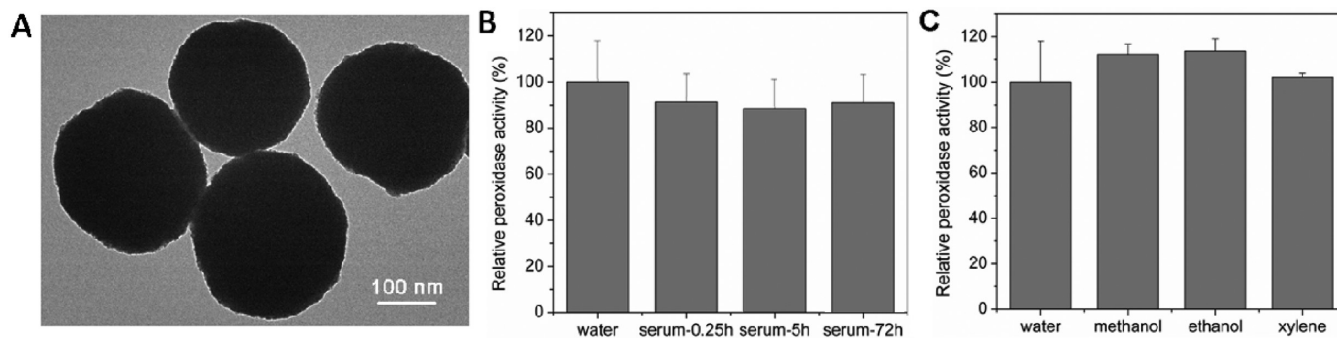


Figure 1. Characterization of dextran-coated MNPs. (A) TEM images of dextran-coated MNPs. (B) Stability of peroxidase activity of dextran-coated MNPs in 70% normal mouse serum at 37 °C over 72 h or (C) in organic chemical reagents at 25 °C over 1 h. $P > 0.05$ from water incubation control; $n = 3$ per group.

67 of nanoparticles and investigated the biodistribution and organ
68 clearance of MNPs in mouse models.

69 ■ MATERIALS AND METHODS

70 **Nanoparticle Synthesis and Characterization.** Dextran-
71 coated MNPs were synthesized according to the hydrothermal
72 method with some modification.²² Briefly, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was
73 dissolved in ethylene glycol, followed by addition of NaAc and
74 dextran. The mixture was stirred vigorously for 30 min, then
75 sealed in an autoclave, and heated at 200 °C for 30 h. The
76 products were collected by washing several times with ethanol
77 and dried at 60 °C.

78 Silica-coated MNPs were prepared following the Stöber
79 method.²³ Briefly, a solution of base (10 M NaOH) was mixed
80 with iron salts at a molar ratio ($\text{FeCl}_2:\text{FeCl}_3$) of 1:2. The
81 mixture was stirred for 1 h at 20 °C and then was heated at 90
82 °C for 1 h. The iron oxide dispersion was stirred for 30 min at
83 90 °C upon addition of 200 mL of trisodium citrate solution
84 (0.3 M). The ultrafine magnetic particles were precipitated with
85 acetone, and the supernatant was decanted by a magnet.
86 Subsequently, water was added to redisperse ultrafine magnetic
87 particles. The resultant dispersion was treated by dialysis and
88 adjusted to 2.0 wt %. The obtained magnetite dispersion was
89 then homogenized by ultrasonic vibration in a water bath.
90 Under continuous mechanical stirring, tetraethoxysilane was
91 slowly added. After 12 h of stirring, silica was formed on the
92 surface of magnetite nanoparticles through hydrolysis and
93 condensation of tetraethoxysilane.

94 A peroxidase activity test was carried out on dextran-coated
95 MNPs and silica-coated MNPs at room temperature. Dextran-
96 coated or silica-coated MNPs (20 μg) were first mixed with 500
97 mM H_2O_2 in sodium acetate buffer (pH 3.5), using 3,3',5,5'-
98 tetramethylbenzidine (TMB, Sigma) as the substrate. Color
99 reactions were recorded 30 min after the addition of substrate.

100 **MNP Biodistribution and Clearance Study.** Male Balb/c
101 mice (6–8 weeks old) were purchased from the Institute of
102 Materia Medica, Chinese Academy of Medical Sciences. All
103 animal studies were performed with the approval of the
104 Chinese Academy of Sciences Institutional Animal Care and
105 Use Committee. Mice were intravenously administered with 10
106 mg/kg of dextran-coated MNPs or silica-coated MNP to
107 evaluate organ distribution of MNPs ($n = 4$ mice per group).
108 PBS-administered mice were used as control. At 0.25, 5, and 72
109 h postinjection, mice were sacrificed. Organs of interest (liver,
110 spleen, lungs, kidneys, lymph node and thymus) were collected,
111 fixed and embedded in frozen or paraffin sections for
112 subsequent histological staining.

The tissue location of MNPs were evaluated by staining 113
tissue sections using MNPs-peroxidase assay. Paraffin-embed- 114
ded tissue sections were first deparaffinized by washing twice in 115
xylene for 10 min and then hydrated progressively in an ethanol 116
gradient. Endogenous peroxidase activity was quenched by 117
incubation with 0.3% H_2O_2 in methanol for 30 min. Freshly 118
prepared DAB was added for color development. The staining 119
was stopped by rinsing sections with double-distilled water. 120
Tissue sections were finally counterstained with hematoxylin. 121
Frozen tissue sections were incubated with 0.3% H_2O_2 to 122
quench endogenous peroxidase activity and then stained with 123
DAB to develop color. After rinsing with water, tissues were 124
counterstained with hematoxylin. Stained tissues were imaged 125
under an Olympus microscope and quantified using ImageJ 126
software. 127

Prussian Blue Staining. Paraffin-embedded tissues from 128
MNP-administered mice were stained by Prussian blue staining 129
solution (a mixture of 20% hydrochloric acid and 10% 130
potassium ferrocyanide solution in a 1:1 volume ratio) and 131
then washed gently with PBS. Tissues were also counterstained 132
with hematoxylin and eosin (H&E) staining. 133

Data Analysis. Quantitative data are expressed as means \pm 134
SD. Means were compared using one-way ANOVA and 135
Student's t test. P values of <0.05 were considered statistically 136
significant. 137

138 ■ RESULTS

Intrinsic Peroxidase Activity of MNPs. To date a wide 139
variety of MNPs have been synthesized, differing in size 140
(hydrodynamic diameter varying from 10 to 500 nm) and type 141
of coating material used, such as dextran, silica, albumin, 142
polyethylene glycol, gold and liposomes.^{24–26} Among various 143
types of coating materials, natural polymers, in particular 144
dextran, are the most popular for coating MNPs because of 145
their biocompatibility and simple coating protocols.^{27–29} 146
Therefore, dextran-coated MNPs were selected as representa- 147
tive for this investigation. 148

The synthesized dextran-coated MNPs were first analyzed by 149
transmission electron microscope (TEM). As shown in Figure 150
1A, nanoparticles were monodispersed and evenly distributed 151
in size with an average diameter of 300 nm. We then 152
characterized the peroxidase activity of dextran-coated MNPs in 153
serum and in organic solutions. Serum incubation at 37 °C was 154
used to test the stability of peroxidase activity of MNPs in 155
circulation in mice. Organic solutions, including ethanol, 156
methanol and xylene, were used for MNP incubation to test 157
the in vitro stability of peroxidase activity of MNPs because 158

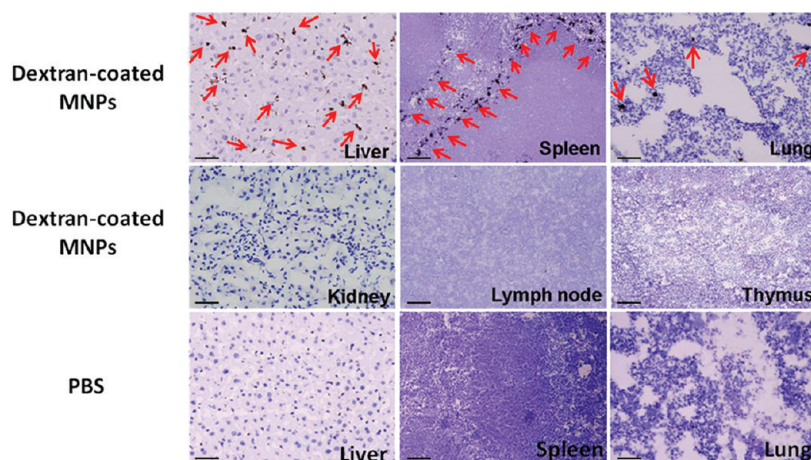


Figure 2. MNPs-peroxidase staining of frozen liver, spleen, lung, kidney, lymph node and thymus from dextran-coated MNP-administered mice or PBS-administered mice. Arrows indicate stained MNPs. (Scale bar = 100 μm .)

159 tissue slides located with the MNPs will be treated by these
 160 chemical reagents during the tissue staining process. After
 161 incubation in serum or organic solutions, MNPs were separated
 162 using a magnet and then mixed with TMB substrate for color
 163 formation. Peroxidase activity of MNPs was indicated by
 164 measuring the formed color intensity. As shown in Figure 1B,C,
 165 the peroxidase activity of dextran-coated MNPs did not show a
 166 substantial change, suggesting peroxidase activity stability over
 167 this period. We next compared the peroxidase activity of
 168 commercially available dextran-coated MNPs with our
 169 laboratory synthesized dextran-coated MNPs. As shown in
 170 Supplementary Figure S1 in the Supporting Information,
 171 commercial MNPs at the same size exhibited the similar
 172 peroxidase activity with our synthesized MNPs under the same
 173 conditions, illustrating that there are no limitations to MNP
 174 sources.

175 **Biodistribution of MNPs.** By utilizing the peroxidase
 176 activity of magnetic nanoparticles that can catalyze the
 177 oxidation of peroxidase substrates to form a color deposition
 178 at the site of MNPs, we evaluated the organ biodistribution of
 179 dextran-coated MNPs in mice. Four mice each intravenously
 180 received an injection of 10 mg/kg of MNPs and then were
 181 sacrificed at 5 h postinjection. PBS-administered mice were
 182 used as control. Organs including liver, spleen, lung, kidney,
 183 heart, lymph node and thymus were harvested and embedded
 184 in frozen sections and stained with MNPs-peroxidase assay
 185 using DAB as staining substrate. As shown in Figure 2, the
 186 dextran-coated MNPs can catalyze the oxidation of DAB in the
 187 presence of H_2O_2 and form an insoluble brown deposition at
 188 the sites of MNPs. To quantify the accumulated MNPs in these
 189 tissues, ten representative tissue slices were randomly chosen
 190 from the same tissue. The accumulated MNPs per area of these
 191 representative slices were analyzed by the ImageJ. As shown in
 192 Supplementary Figure S2 in the Supporting Information,
 193 dextran-coated MNPs were mainly localized in liver, spleen
 194 and lung. Rare uptake of MNPs was observed in kidney, lymph
 195 node and thymus. No stains were observed in PBS-
 196 administered control tissues, indicating that no endogenous
 197 peroxidase activity interferes MNPs-peroxidase staining.

198 We next examined the cellular location of MNPs in liver,
 199 spleen and lung. After MNPs-peroxidase assay staining, tissue
 200 sections were further stained by H&E. As shown in Figure 3,
 201 MNPs were distributed mainly in Kupffer macrophage cells in
 202 liver, alveolar macrophages in lung and macrophage peri-

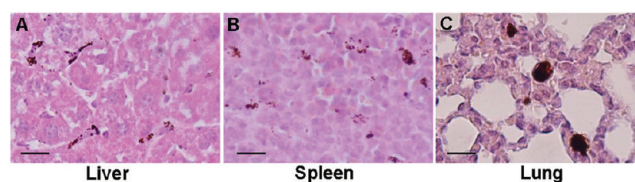


Figure 3. Dextran-coated MNPs were uptaken mainly by reticuloendothelial system (RES) in liver, spleen and lung. Tissue sections were first stained by MNPs-peroxidase assay and then counterstained with H&E to make cell structure more visible. (Scale bar = 50 μm .)

follicular areas in spleen, demonstrating that the accumulation
 of MNPs in these organs is caused mainly by reticuloendothelial cell uptake, which is consistent with previous reports.³⁰

To validate the feasibility of MNPs-peroxidase assay staining
 in paraffin-embedded tissues, MNP-administered mice were
 sacrificed at 5 h postinjection and the main organs were
 collected and paraffin-embedded for MNPs-peroxidase staining.
 As shown in Figure 4, paraffin-embedded organs show a

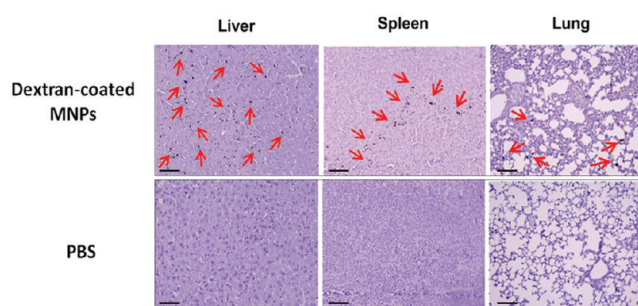


Figure 4. MNPs-peroxidase staining of paraffin-embedded liver, spleen, and lung from dextran-coated MNP-administered mice (upper) or PBS-administered mice (lower). Arrows indicate stained MNPs. (Scale bar = 100 μm .)

distinct localization of MNPs. These results indicate that MNPs
 can be detected by the MNPs-peroxidase approach both in
 frozen and in paraffin-embedded organs. Importantly, although
 MNPs were subjected to xylene deparaffinization, ethanol
 rehydration and quenching endogenous peroxidases in
 methanol, the peroxidase activity of MNPs has not been
 influenced, as evident in Figure 1C. We further showed that

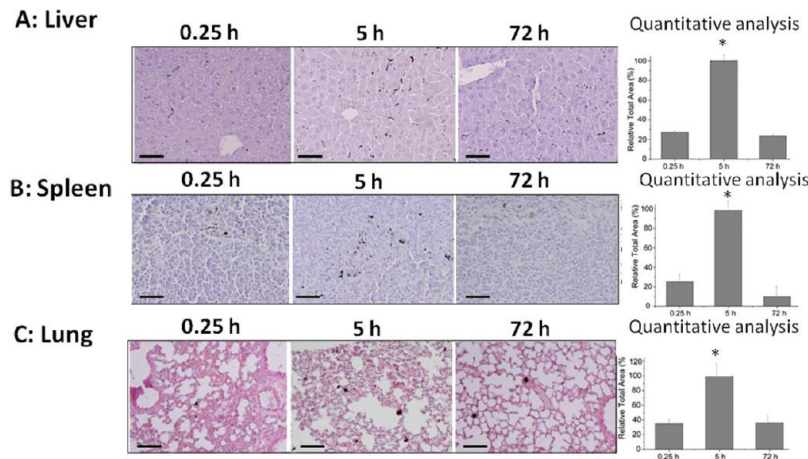


Figure 5. Organ clearance of dextran-coated MNPs. Liver, spleen, and lung were acquired from mice at 0.25, 5, and 72 h postinjection of 10 mg/kg of MNPs. Quantitative analysis of MNPs in liver, spleen, and lung was made using ImageJ software. *, $P < 0.05$ from the time point of 0.25 h postinjection of MNPs; $n = 4$ mice per group. (Scale bar = 100 μm .)

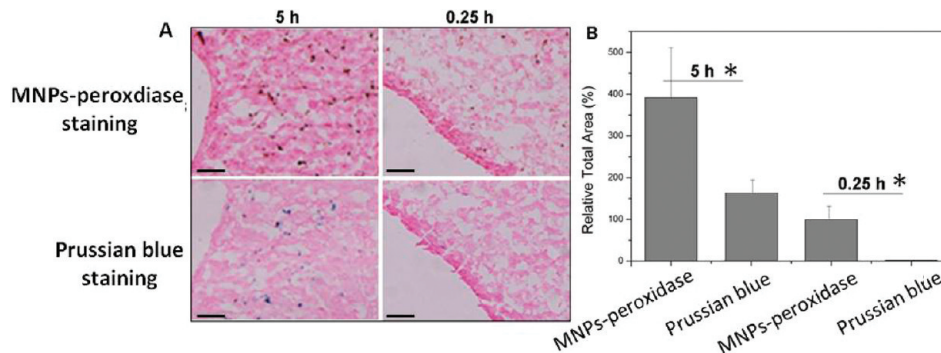


Figure 6. Comparison of the sensitivity of MNPs-peroxidase staining and Prussian blue staining. (A) Staining images of liver by MNPs-peroxidase staining or Prussian blue. Liver sections were acquired from mice at 0.25 and 5 h after intravenous injection of 10 mg/kg of dextran-coated MNPs. (B) Quantitative analysis was made using ImageJ software. *, $P < 0.05$. (Scale bar = 100 μm .)

218 administered MNPs at different sizes all can be visualized
219 clearly in tissues (Supplementary Figure S3 in the Supporting
220 Information), indicating that there are no limitations to
221 MNP sizes.

222 **Organ Clearance of MNPs.** Clearance of MNPs in organs
223 was determined in dextran-coated MNPs-administered mice.
224 The main organs were harvested at 0.25, 5, and 72 h
225 postinjection, embedded in paraffin sections and cut at 5 μm
226 for the subsequent histological staining. Ten representative
227 slices were randomly chosen from each organ. After tissue
228 staining by MNPs-peroxidase assay, the accumulated MNPs per
229 area of these representative slices were analyzed by ImageJ. The
230 error bars from these ten representative tissue slices are within
231 10%, indicating that the change of accumulated MNP slice by
232 slice is not substantial in the same organ. Therefore, the
233 measured MNPs of a random ten slices can totally represent
234 the MNP accumulation in these organs. The final data were the
235 means of four mice per group. As shown in Figure 5, the uptake
236 of MNPs in liver, spleen and lung increased steadily from 0.25
237 to 5 h postinjection and then decreased from 5 to 72 h
238 postinjection, indicating a rapid clearance.

239 **Sensitivity Comparison with Prussian Blue Staining.**
240 Prussian blue staining is commonly used for iron oxide MNP
241 staining in tissues.^{12–14} However, the Prussian blue staining
242 method is not sensitive enough when detecting low amounts of
243 MNPs. In addition, the results are usually affected by the

loosely bound endogenous ferric iron in tissues.¹⁵ The MNPs-
244 peroxidase approach does not have this issue because the
245 endogenous iron exhibits little peroxidase activity.¹ We
246 compared the sensitivity of MNPs-peroxidase staining and
247 Prussian blue staining by detecting two sequential tissue
248 sections using these two approaches. As shown in Figure 6,
249 MNPs-peroxidase staining visualized two times more MNPs
250 than Prussian blue staining at two sequential tissue sections.
251 And when MNP amount in organs is too low to be detectable
252 by Prussian blue staining, MNPs-peroxidase staining still can
253 locate them out (Figure 6A, right column), suggesting a better
254 detection sensitivity of MNPs-peroxidase approach than
255 Prussian blue staining.
256

257 **Detection of Administered Silica-Coated MNPs in**
258 **Tissues.** Silica has also been commonly used as coating
259 materials for MNPs because silica coating provides an ideal
260 platform for drug delivery.^{31–33} We next examined the
261 clearance of silica-coated MNPs by MNPs-peroxidase staining.
262 Silica-coated MNPs at 10 mg/kg were first injected intra-
263 venously into the mouse. The mouse was then sacrificed at
264 0.25, 5 and 72 h postinjection, and the organs were harvested
265 and embedded in paraffin sections. To quantify the
266 accumulated MNPs in these tissues, ten representative tissue
267 slices were randomly chosen from the same organ. After tissue
268 staining by MNPs-peroxidase assay, the accumulated MNPs per
269 area of these representative slices were analyzed by ImageJ. The

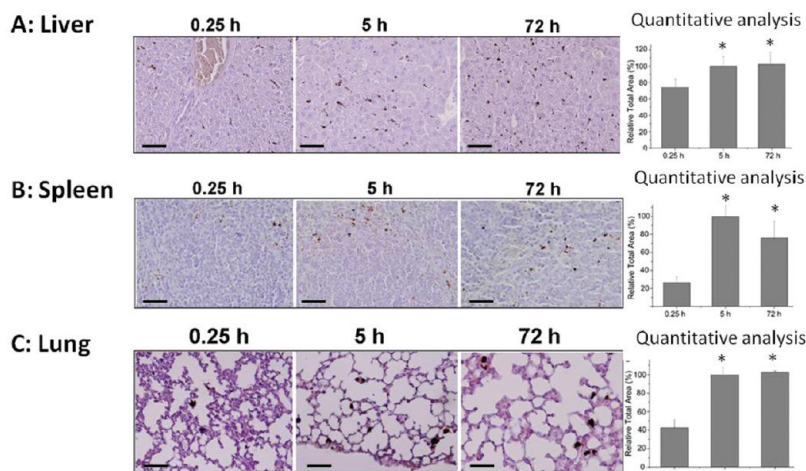


Figure 7. Organ clearance of silica-coated MNPs. Liver, spleen, and lung were acquired from mice at 0.25, 5, and 72 h postinjection of 10 mg/kg of MNPs. Quantitative analysis of MNPs in liver, spleen, and lung was made using ImageJ software. *, $P < 0.05$ from time point of 0.25 h postinjection of MNPs; $n = 4$ mice per group. (Scale bar = 100 μm .)

270 final data were the means of four mice per group. As shown in
 271 Figure 7, the uptake of silica-coated MNPs in liver, spleen and
 272 lung increased steadily from 0.25 to 72 h, suggesting a rather
 273 slow clearance of silica-coated MNPs from the body. The
 274 results are consistent with the previous reports which
 275 demonstrated that silica-coated nanoparticles were cleared out
 276 from the body over several days.³¹

277 ■ DISCUSSION

278 Researchers employ numerous techniques to study MNP
 279 behavior in mice, including in vivo molecular imaging,^{7–9} ex
 280 vivo organ biodistribution of indicator labeled nanoparticles
 281 following necropsy and histology staining.^{34,35} The recent
 282 advance of commercially available small-animal molecular
 283 imaging instrumentation, such as single-photon emission
 284 computed tomography (SPECT), positron emission tomog-
 285 raphy (PET) and optical imaging, has enabled in vivo, dynamic,
 286 quantitative measurements of radio- or fluorophore-labeled
 287 MNPs in mouse models.^{7–9} However, exogenous labeling
 288 generally changes the surface properties of MNPs and increases
 289 hydrodynamic diameter, which can interfere with their in vivo
 290 behavior. Thus developing an MNP intrinsic property-based
 291 method could offer the possibility to provide a real under-
 292 standing of their behavior after administration into the body.

293 Previously, we reported for the first time that MNPs exhibit
 294 peroxidase activity that could catalyze the oxidation of
 295 peroxidase substrates to produce a color reaction,¹ and since
 296 that time numerous papers on this topic have been
 297 published.^{16–21} In this present study, we are developing an ex
 298 vivo MNPs detection method based on their intrinsic
 299 peroxidase activity. This MNPs-peroxidase approach was
 300 designed to visualize administered MNPs in tissues by
 301 catalyzing the oxidation of peroxidase substrates to form a
 302 color deposition at the site of MNPs. We first established that
 303 the peroxidase activity of MNPs remained stable after
 304 incubation in serum at 37 °C or in organic solution at 25 °C
 305 (Figure 1), which is necessary because an intrinsic property
 306 under consideration for MNP detection in mice must be
 307 sufficiently stable when MNPs are in circulation in organs and
 308 throughout their passage across the in vitro tissue staining
 309 process that involves organic solution incubation. We next
 310 demonstrated that commercially available MNPs exhibited

similar peroxidase activity with laboratory synthesized MNPs at
 311 the same size (Supplementary Figure S1 in the Supporting
 312 Information), illustrating that there are no limitations to MNP
 313 sources. Thereafter, main organs from MNP-administered mice
 314 were collected for MNPs-peroxidase assay staining. As shown in
 315 Figures 2 and 4, the administered MNPs are evident in both
 316 frozen and paraffin tissue sections, confirming the feasibility of
 317 visualizing MNPs in tissues by employing their intrinsic
 318 peroxidase activity. The endogenous iron species were not
 319 visualized with the same staining process when peroxidase
 320 substrate was added (Figures 2 and 4, lower) because the
 321 endogenous iron species do not exhibit intrinsic peroxidase
 322 activity due to the differences of the mineral phase composition.
 323 We then showed that administered MNPs at different sizes all
 324 can be visualized clearly in tissues (Supplementary Figure S3 in
 325 the Supporting Information), indicating that that there are no
 326 limitations to MNP sizes. Finally, the MNP clearance study
 327 provided quantitative information on MNP accumulation over
 328 time in several main organs (Figure 5), which further confirms
 329 the feasibility of the MNPs-peroxidase approach in MNP
 330 detection in tissues. Prussian blue staining images show
 331 obviously less sensitive measurement for MNPs as compared
 332 with the MNPs-peroxidase approach in sequential tissue
 333 sections (Figure 6), which can be explained by the highly
 334 effective catalytic activity of MNPs.¹ 335

336 ■ CONCLUSION

337 In conclusion, we have demonstrated the feasibility of using the
 338 MNPs-peroxidase approach to detect MNPs in mice by
 339 employing their intrinsic peroxidase-mimicking activity. As
 340 shown above, the MNP biodistribution and clearance in mouse
 341 models were determined conveniently by visualizing and
 342 quantifying MNP localization within the main organs using
 343 their peroxidase activity. The developed MNPs-peroxidase
 344 approach is more sensitive when compared with the traditional
 345 Prussian blue staining method because of the highly effective
 346 catalytic activity of MNPs. Importantly, as this approach avoids
 347 complex and costly labeling with exogenous indicators, this
 348 reduces false background signals and provides the possibility to
 349 understand the real behavior of MNPs in mice and thus has
 350 significant implications for the clinical translation of MNPs.

351 Presumably, other nanoparticles having intrinsic peroxidase
352 activity could also be considered.

353 ■ ASSOCIATED CONTENT

354 ● Supporting Information

355 Additional experimental details and related figures. This
356 material is available free of charge via the Internet at [http://](http://pubs.acs.org)
357 pubs.acs.org.

358 ■ AUTHOR INFORMATION

359 Corresponding Author

360 *X.Y.: Institute of Biophysics, Chinese Academy of Sciences,
361 National Laboratory of Biomacromolecules, Datun Road No.
362 15, Beijing 100101, China; e-mail, yanxy@ibp.ac.cn; tel, +86 10
363 6488 8583; fax, +86 10 6488 8584. M.L.: e-mail, [moon.ibp.ac.cn](mailto:mmliang@
364 <a href=); tel, +86 10 6488 8503; fax, +86 10 6488 8584.

365 Notes

366 The authors declare no competing financial interest.

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