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Photochemistry on the Space Station-Antibody Resistance to Space Conditions after Exposure Outside the International Space Station

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Abstract :

Antibody-based analytical instruments are under development to detect signatures of life on planetary bodies. Antibodies are molecular recognition reagents able to detect their target at sub-nanomolar concentrations, with high affinity and specificity. Studying antibody binding performances under space conditions is mandatory to convince space agencies of the adequacy of this promising tool for planetary exploration.

To complement previous ground-based experiments on antibody resistance to simulated irradiation, we evaluate in this paper the effects of antibody exposure to real space conditions during the EXPOSE-R2 mission outside the International Space Station. The absorbed dose of ionizing radiation recorded during the 588 days of this mission (220 mGy) corresponded to the absorbed dose expected during a mission to Mars. Moreover, samples faced, at the same time as irradiation, thermal cycles, launch constraints, and long-term storage. A model biochip was used in this study with antibodies in freeze-dried form and under two formats: free or covalently grafted to a solid surface.

We found that antibody-binding performances were not significantly affected by cosmic radiation, and more than 40% of the exposed antibody, independent of its format, was still functional during all this

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experiment. We conclude that antibody-based instruments are well suited for in situ analysis on planetary bodies.

Keywords : Astrobiology, Cosmic rays, Biochip, Antibody, Planetary exploration

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51 **Introduction**

52 In the context of planetary exploration, the use of miniaturized instruments based on target-
53 binding reagent with high specificity and binding affinity is clearly relevant. Indeed, such
54 instruments can, in principle, detect unambiguously thousands of different targets from small
55 molecules like amino acids to complex molecules and microorganisms in a single assay. The
56 basic principle can be summarized as follow: the target-binding reagents, like antibodies (Ab),
57 are fixed on a solid surface to specifically capture their target. In direct methods, target
58 interaction with the binding site of Ab and the use of appropriate reagents generate a visible
59 signal capable of being measured, for instance, by a charge coupled device (CCD) camera. In
60 the field of astrobiology, several Ab-based biochips have been proposed as miniaturized high
61 throughput detection systems, to detect biomarkers, especially organic ones, in the search for
62 extraterrestrial life (Parro *et al*., 2005; Le Postollec *et al.,* 2007; Parro *et al.,* 2008; Martins *et*
63 *al.,* 2011; Parro *et al.,* 2011a, Parro *et al.,* 2011b; Sims *et al*., 2012; McKay *et al.,* 2013; Smith
64 *et al.,* 2014).

65 However, space is a hazardous environment in particular due to strong irradiation from primary
66 and secondary particles produced by Galactic Cosmic Rays (GCR) and Solar Energetic Particles
67 (SEP), temperature variations and long duration storage (during the cruise phase especially).
68 As a consequence, one main concern relies on the resistance of biochips to these cumulative
69 space conditions. In recent years, studies have been performed to evaluate the resistance of Ab
70 to specific space conditions, especially regarding the effect of some ionizing particles on Ab
71 binding performances (Le Postollec *et al.,* 2009a; Le Postollec *et al.,* 2009b; Baqué *et al.,* 2011;
72 de Diego-Castilla *et al.,* 2011; Baqué *et al*., 2017; Coussot *et al.,* 2017). However, these ground-
73 based simulations were limited due to the use of one single type and energy particle at a time
74 and the experimental characteristics of the beam (high fluxes in a short exposure time,

75 unidirectional irradiation). In the space environment, biochips would be exposed to continuous
76 and sporadic fluxes of various particles in a large range of energies and all directions,
77 concomitantly with other parameters inherent to a space mission including thermal variations,
78 vibrations and storage conditions.

79 To complement ground-based studies, Derveni *et al.* conducted in 2007 a 12 days mission on
80 the BIOPAN-6 low Earth orbit platform to demonstrate the effects of cumulative irradiations
81 on two antibodies' ability to bind to their respective antigens (Derveni *et al.,* 2012; Derveni *et*
82 *al.,* 2013). The absorbed dose recorded at the samples position by the dosimeters was 2.4
83 milligrays (mGy) during the experiment. Both freeze-dried Ab, in free format and absorbed into
84 laser-cut glass fiber pads, were reported as unaffected with regard to their target-binding
85 abilities after their low Earth orbit platform exposure. Analyses were done with conventional
86 indirect enzyme-linked immunosorbent assays (ELISA). However, major loss of Ab activities
87 (>70%) was pointed out and explained by the Ab preparation procedure, in particular the freeze-
88 drying step and storage time period. These issues complicated the interpretation of the results.
89 This study highlighted that using Ab as target-binding reagent necessitates controlling its ability
90 to bind to its target (refers also as its functionality) after each step of the antibody-based device
91 development. Consequently, for biochips, a control is required after the Ab immobilization onto
92 the solid surface (to determine the initial rate of functional Ab), but also after washing, freeze-
93 drying, storage, and rehydration steps. These controls permit to precisely quantify the number
94 of Ab that remains functional after the preparation procedure and before undergoing some
95 putative deleterious experiments.

96 In the frame of the BiOMAS project (Biochip for Organic Matter Analysis in Space), we
97 developed a model biochip, in which a specific Ab (anti-horseradish peroxidase Ab, quoted
98 anti-HRP Ab hereafter) was used in free format or covalently immobilized onto a solid surface.

A direct detection method called “Antibody Anti-HorseRadish Peroxidase (A2HRP)” was developed and fulfilled the validation criteria requested by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the validation of bioanalytical methods (Coussot *et al.,* 2018a, Coussot *et al.,* 2018b).This gold standard method permits to evaluate accurately and precisely the number of free or immobilized functional Ab (interday variation <12.1%). The Lower Limit of Quantification (LLOQ) corresponds to 1.40 ± 0.18% of the initial rate of active Ab. The A2HRP method was successfully used in optimizing freeze-drying conditions to better preserve the Ab functionality, notwithstanding its format (Coussot *et al.*, 2018c), and in evaluating the Ab resistance under various stressful environments (Coussot *et al.,* 2017, Coussot *et al.*, 2018c).

In this work, we first summarize our experiment (see also Vigier *et al.* 2013), which was part of the Photochemistry on the Space Station (PSS) project (Cottin *et al.*, 2015) and the analytical developments we conducted prior to the final analyses performed on the samples few weeks after their return to Earth (Coussot *et al.* 2018a, 2018b and 2018c). Both formats, free anti-HRP Ab and anti-HRP Ab immobilized onto a surface, have been conditioned into a homemade sample holder unit and installed outside the International Space Station (ISS) on the EXPOSE-R2 platform. We then present the results on the resistance of our model biochip against a long-time exposure (more than 18 months) to real space conditions. The relevance of antibody-based biochips for space exploration is finally discussed.

120 **I. Materials and methods**

121 ***II.1 Chemical, reagents and materials***

122 Mouse monoclonal anti-horseradish peroxidase antibodies (anti-HRP Ab) were obtained from
123 MyBioSource (clone number B215M, batches 2F17811 & 2F15911, USA). Bovine serum
124 albumin (BSA, fraction V, 96–100% protein, batch SLB38588V), Horseradish peroxidase
125 (HRP, EC 1.11.1.7, batch SLBF8268V, with a purity index of 1.9), Tween® 20 (impurities
126 ≤3% in water), o-phenylenediamine dihydrochloride (OPD-2HCl), hydrogen peroxide (H_2O_2,
127 30% (v/v) solution equivalent to a titer of 120V of oxygen (10.73 M) upon opening (Coussot *et*
128 *al.*, 2018c)), Dulbecco's Phosphate Buffered Saline (DPBS, 10X solution, pH 7.4, used in a
129 final concentration of 1X in water solvent), sulfuric acid (H_2SO_4, ≥97,5%), citric acid (≥
130 99,5%), sodium hydroxide solution (10M solution for molecular biology), sodium phosphate
131 dibasic dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, ≥ 99,0%), sodium bicarbonate ($NaHCO_3$, ≥ 99,5%),
132 sodium carbonate (Na_2CO_3, ≥ 99,5%), D(+)-saccharose (sucrose, ≥ 99,0%), L-Histidine (His,
133 ≥ 99,0%), D-Arginine (Arg, ≥ 99,0%), ethanol (EtOH, ≥ 99.5%), hydrochloric acid (HCl, 37%),
134 were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Corning DNA Bind™
135 8-well strip plate with N-hydroxysuccinimide modified surface (NHS-wells) were provided by
136 Sigma Aldrich (Saint-Quentin Fallavier, France). NHS-wells were manufactured by Air
137 Liquide (Sassenage, France) to fit perfectly with the shape and size of EXPOSE-R2 closed cells
138 provided by the French Space Agency (CNES, Toulouse) (Vigier *et al.,* 2013), called simply
139 "cells" in the following. These home-designed NHS-wells had a diameter of 8.4 mm with a 7.1
140 mm height. Teflon cap were specially manufactured by Air Liquide to close these custom
141 designed NHS-wells (Coussot *et al.*, 2018d). The ultra-pure water was obtained from a
142 Millipore Purification system. Other chemicals are analytical grade and used as received.
143 During the assays, the relative humidity and air temperature were controlled using a

144 thermohygrometer Testo 605-H1 (Type 05600610, identification number 39227197/205),
145 equipment whose calibration is traceable to national standards (certificate number 1306125).
146 During all assays, room temperature was 23.2±1.3°C, and recorded relative humidity (RH) was
147 25.7±0.9%.

148

149 ***II.2 Sample preparation***

150 Long-time exposure to real space conditions during the EXPOSE-R2 mission were evaluated
151 on freeze-dried Ab in both covalently immobilized format (labelled "G" *for grafted*) and in-
152 solution Ab format (free anti-HRP Ab labelled "F" *for free*). The immobilization strategy, the
153 freeze-drying optimized procedure, the A2HRP validated protocol were detailed elsewhere
154 (Coussot *et al.,* 2017, Coussot *et al.,* 2018a, Coussot *et al.,* 2018b, Coussot *et al.*, 2018c). The
155 freeze-drying stock solution was freshly prepared and was composed of 1.05 g of citric acid
156 (0.1M), 38.75 mg of His (5 mM), 52.5 mg of Arg (5 mM) in a final volume of 50 mL of water
157 and adjusted to pH 6.5 with NaOH (10M) before adding 10 µL of Tween®20.

158

159 *II.2a Preparation of anti-HRP Ab in free format*

160 The home-designed NHS-wells were filled up with 250 µL of BSA (3% (w/v) in DPBS (1X)).
161 After overnight reaction time at room temperature (RT) in the dark, the wells were emptied and
162 rinsed three times with DPBST solution (DPBS (1X) with 0.05% (v/v) Tween®20) and three
163 times with DPBS. An anti-HRP Ab solution was directly prepared at 200 µg.mL^{-1} in the above
164 freeze-drying solution immediately before deposit into the BSA-saturated home-designed
165 NHS-wells (noted also as inactivated BSA wells). In each inactivated BSA well, a volume of
166 100 µL of the anti-HRP Ab in the freeze-drying solution was mixed with 20 µL of a freshly
167 prepared sucrose solution (5g.L^{-1} in water). The wells were placed within the homemade pre-

168 frozen aluminium block designed by our group, and kept frozen by adding liquid nitrogen to
169 follow the optimized freeze-drying procedure (Coussot *et al.*, 2018c) as described in the section
170 ***II.3***.
171
172 *II.2b Preparation of anti-HRP Ab in covalently immobilized format*
173 The home-designed NHS-wells were filled up with a 200 µg.mL^{-1} of anti-HRP Ab solution in
174 DPBS (1X). After a minimum of 4h under gentle agitation at RT, a saturation step with 250 µL
175 of BSA solution (3% w/v in DPBS) per well was carried out to prevent non-specific bindings
176 (Baqué *et al.* 2011), followed by 3 washings with DPBST solution, and 3 washings with PBS.
177 In each covalently immobilized and saturated Ab wells were added 100 µL of the freeze-drying
178 solution and 20 µL of a freshly prepared sucrose solution (5g.L^{-1} in water). Samples were then
179 freeze-dried using the homemade aluminium holder as described in the section ***II.3***.
180

181 ***II.3 Freeze-drying procedure***

182 The aluminium holder had the advantage of running multiple samples at the same time avoiding
183 freeze-drying batch effects. As described elsewhere, this device was pre-frozen and transferred
184 into the central part of a freeze-dryer (Christ Alpha 2–4 from Martin Christ GmbH, Germany)
185 (Coussot *et al.*, 2018c). Freeze-drying was performed overnight (condenser temperature −85°C,
186 vacuum 0.05 mbar). When the freeze-drying process was achieved, the chamber of the freeze-
187 dryer was filled with nitrogen gas before closing the sample-containing aluminium holder,
188 which kept the samples hermetically sealed and sheltered them from the moisture and light until
189 its opening. The opening of the aluminium holder was done in a glove box under a controlled
190 atmosphere of helium (10% He) in argon (Ar) provided by Air Liquide (Sassenage, France) to
191 maintain a RH level of 10% to 15%. During our experiments in the glove box, the RH and air

192 temperature were controlled using a thermohygrometer Testo 605-H1 (Testo, France). RH was
193 12±3% and air temperature was 22.8±2.3°C. After opening the aluminium holder, freeze-dried
194 samples were capped and directly transferred into the CNES closed cells using a tool vacuum
195 suction pen (FFQ939 from Mayf's Online Shop, China). Into each cell, two
196 ThermoLuminescent Dosimeters (TLDs) were placed at the bottom of the cell, below the
197 samples. These passive dosimeters were analyzed at the end of the mission. The absorbed dose
198 measured into the cells was 220 mGy that corresponds to the dose that can be expected during
199 a mission to Mars including 8 months travel and 18 months at Mars surface. (Hassler *et al*.,
200 2014; Coussot *et al*., 2018d). The cells were then screwed with a final tightening of 0.7 Nm
201 using a torque screwdriver TorqueVario®-S (Wiha, Germany) (Vigier *et al.,* 2013; Coussot *et*
202 *al*., 2018d).

203

204 ***II.4 Experiment outside the International Space Station (ISS)***

205 To study the impact of all constraints encountered by a biochip during an entire space mission
206 (long duration storage, transportation, take-off/landing shocks, thermal constraints, and
207 cumulative effects of cosmic rays particles), 41 anti-HRP Ab samples (21 anti-HRP Ab in the
208 free format "F" and 20 in the covalently immobilized format "G") took part of the "Biochip in
209 PSS experiment" (Vigier *et al.,* 2013; Cottin *et al.,* 2015) during the EXPOSE-R2 mission. An
210 overall view of EXPOSE-R2 mission is illustrated in **Figure 1**. All samples (14 exposed on ISS
211 and 27 kept on Earth) were simultaneously prepared, within the same freeze-drying batch, as
212 described in the experimental section ***II.3***. The 14 exposed cells were spread on the 2 exposure
213 levels of the Tray 3 of the EXPOSE carrier dedicated to the PSS experiment (4F and 3G anti-
214 HRP Ab in each level). Disposition of the exposed cells on the sample carrier was the same for
215 the upper and the lower level of the tray 3 (denoted as upper tray and lower tray, respectively).

216 Launch to ISS (July 23th, 2014) and storage inside ISS was at ambient temperature (22-25°C
217 on average) (Rabbow *et al.,* 2017). Then, the EXPOSE-R2 platform was placed outside the ISS
218 on the Universal platform D on August 18th with opening of the valves for venting the inner
219 part of EXPOSE-R2 on August 20th, and removal of the UV shield in October 22nd, 2014. On
220 February 3rd, 2016 trays were covered and brought back inside the ISS. Thus, the exposed
221 samples spent 566 days outside the ISS over the 588 days of the mission (1 year, 8 months).

222 During their extravehicular exposition, the samples on Tray 3 were submitted to radiations and
223 temperatures varying between -20.9 °C and 57.98 °C (Rabbow *et al.,* 2017). On March 2nd 2016,
224 EXPOSE-R2 Tray 3 landed on Earth. During transit from the Baïkonur cosmodrome to
225 Moscow, and from Moscow to the German Aerospace Center (DLR, Germany) a recording of
226 temperatures was done, the temperatures oscillated between 20°C and 24°C, then from DLR to
227 LISA (Laboratoire Interuniversitaire des Systèmes Atmosphériques, Créteil, France) (Rabbow
228 *et al.*, 2017), and from Créteil to Montpellier a controlled chamber at 4°C was used during
229 transportation in the summer period. De-integration of the exposed cells from the sample
230 carriers was done according to MUSC/DLR/ESA/CNES internal procedures (Rabbow *et al.*,
231 2017). On June 24th 2016, the soldered joint of all the cells (including ground references) was
232 removed, using a mechanical lathe and a manufactured CNES tool, in the mechanics department
233 of the Montpellier University (Coussot *et al.,* 2018d). Samples were immediately sealed in a
234 FoodSaver™ bag (Fischer Scientific, France) and stored in the dark at 4°C until analysis with
235 the A2HRP method (section ***II.7***)

236

237 ***II.5 EXPOSE-R2 mission ground references***

238 During the same period, experiments were performed on ground to control the long-term
239 behaviour of anti-HRP Ab upon storage but not exposed to space conditions. A total of twenty-
240 seven mission ground controls were studied. Six ground cells (3F and 3G anti-HRP Ab) were
241 stored at the French Space Agency (CNES, Toulouse) by maintaining an accurate temperature
242 control at 3.9°±0.8°C over that period. Twenty-one ground cells were kept in DLR (Cologne,
243 Germany): six ground cells (3F and 3G anti-HRP Ab) were stored at 5°C and eight ground cells
244 (4F and 4G anti-HRP Ab) underwent the same thermal history as ISS samples (DLR ΔT), and
245 seven ground samples (4F and 3G anti-HRP Ab) were stored in conditions combining long time
246 storage, varying thermal environment as ISS ones, and UV radiations (DLR ΔT+UV) (Rabbow
247 *et al.,* 2017). All of these 27 ground cells were brought back to Montpellier to be analyzed
248 simultaneously with the 14 exposed cells during all the desoldering process and 4°C storage
249 before rehydration. For the free format, rehydration was done with 100 µL of water and 20 µL
250 of carbonate-bicarbonate buffer (0.1M, pH 9.2) per well in order to fix the pH of the sucrose-
251 containing freeze drying Ab solution to 7.4 to preserve Ab functionality. Free Ab were then
252 covalently coupled to NHS-wells following the procedure described in section ***II.2b***. For the
253 grafted format, rehydration was carried out with 120 µL of DPBS. The rate of functional Ab in
254 both formats was determined with the A2HRP method (section ***II.7*, Figure 1C)**.

255 ***II.6 Additional ground thermal cycling experiments***

256 The anti-HRP Ab were freeze-dried in both formats as described in section ***II.3.*** Thermal
257 cycling experiments were carried out on freeze-dried samples in their sealed bags in a
258 temperature test chamber (Vötsch VT 4004). Two independent thermal variation experiments
259 were done: one with a sharp rise in temperature (5°C/min) reaching a one-hour plateau at 80°C
260 (referred as "80°C peak" in the text), and a long cycling period (178.8 hours or 7.45 days)
261 mimicking thermal variations of exposed cells (see Coussot *et al.*, 2018d) with an amplitude of

262 about 70°C (referred as "long cycle" in the text). Samples were rehydrated as described above
263 and analyzed with the A2HRP method.

264

265 ***II.7 Validated A2HRP method and data interpretation***

266 For all ground-based experiments and that of the overall ISS mission, the A2HRP method
267 **(Figure 1C)** was used to evaluate the functionality of anti-HRP Ab towards its antigen HRP.
268 Briefly, the principles are as following: once the anti-HRP Ab were immobilized onto the NHS-
269 wells to reach a density of 3.9×10^{11} antibody per mm^2 (section ***II.2b***)(Moreau *et al.,* 2011), a
270 HRP stock solution (1 $g.L^{-1}$) was prepared by dissolving the enzyme in a stabilizing solution
271 composed of 0.1 M Na_2HPO_4 and 0.05M citric acid, pH 5.2. A HRP working solution at 200
272 $\mu g.mL^{-1}$ was prepared immediately before use from the HRP stock solution by dilution in
273 DPBS. A volume of 110 µL of the HRP working solution was pipetted into wells containing
274 the previously grafted anti-HRP antibody to saturate all of its binding sites (Moreau *et al.,*
275 2011). At least 2 hours at RT or overnight incubations were considered for maximal binding of
276 HRP to anti-HRP Ab surfaces. Unbound HRP was removed by rinsing 3 times with DPBST
277 and 3 more times with DPBS. The wells were then incubated with a freshly prepared *o*-
278 phenylenediamine dihydrochloride/hydrogen peroxide (OPD/H_2O_2) solution. The OPD/H_2O_2
279 reaction solution was composed of 1 mL OPD stock solution at 5 $g.L^{-1}$ in deionized water, 8.6
280 mL of stabilizing solution described above, and 400 µL of 30% H_2O_2. 50 µL of this OPD/H_2O_2
281 reaction solution and 50 µL of stabilizing solution were mixed into each well. The
282 HRP/OPD/H_2O_2 reaction that converts OPD to 2,3-diaminophenazine (DAP) was then stopped
283 after 4 min by adding 30 µL of STOP solution (H_2SO_4, 4N, pH<1). The HRP-catalyzed OPD
284 oxidation into DAP was recorded at 490 nm with an Infinite 200™ absorbance microplate
285 reader from Tecan (Lyon, France). The activity of the Ab surfaces is expressed as a percentage

286 of the sample/reference ratio of its net absorbance values. The net absorbance values correspond
287 to the measured assay values minus the mean absorbance of the blank obtained with inactivated
288 BSA-wells (section ***II.2a***).
289 All data were reported as the mean ± the standard deviation (SD) from at least three replicate
290 experiments. Statistical significance of the assays was determined using Student's *t* test
291 (p=0.05).
292

293 ## III. Results

294 Stress affects Ab performances in different ways depending on the type and duration of the
295 event (Coussot *et al.*, 2018b; Coussot *et al.*, 2018c). Irradiation effects on anti-HRP Ab was
296 assessed here by evaluating the functionality of the anti-HRP Ab before and after the stress
297 event or by comparing the activity of exposed Ab samples to that of non-exposed ones (named
298 ground references (GR) or controls, see above). Analyses were done according to the validated
299 A2HRP method (**Figure 1C**). The A2HRP method was demonstrated to have the potential for
300 analyzing the binding ability of the anti-HRP Ab in both formats even if deleterious events
301 occurred on the anti-HRP Ab before its coupling to the surface (Coussot *et al.*, 2018c). This
302 direct assay permits a precise and reliable quantitation of HRP bound on the immobilized Ab
303 surface, and consequently to evaluate the anti-HRP Ab binding capacities. It has also
304 demonstrated its suitability to evaluate discrepancies in Ab bindings after their exposure to
305 short-term irradiation events or forced degradation studies (Baqué *et al.* 2017, Coussot *et al.*,
306 2017; Coussot *et al.*, 2018a; Coussot *et al.*, 2018b; Coussot *et al.*, 2018c). In the present paper,
307 we evaluate the Ab resistance after their exposure to real space conditions, and thus for the first
308 time the effect of cumulative and long-term stress events on the performance of a grafted Ab.
309 In order to broadly represent any future Ab-based biochip instruments, we evaluated in a second
310 part, free Ab format since both free and grafted Ab are considered for future applications in
311 planetary exploration.
312

313 ### *III.1. Resistance of anti-HRP Ab in its grafted format after exposure outside the ISS*

314 Since radiation gradients were observed on the EXPOSE platform in previous ISS missions
315 (Berger *et al.*, 2012), the 14 exposed Ab "Flight samples" were spread on the 2 exposure levels
316 of the Tray 3 of the EXPOSE carrier dedicated to the PSS experiment, but with the same

317 disposition on the upper and the lower level of the carrier. All the experiments performed on
318 grafted Ab are reported in **Figure 2**. To only take into account the effects encountered during
319 the ISS mission (flights, extravehicular exposure, and all transportations during the mission),
320 the percentage of functional Ab is calculated using the freeze-dried ground samples stored at
321 5°C in DLR facility (*n*=2 replicates) during all the duration of the mission. At first view, the
322 results reveal a possible degradation of several samples including flight samples. Higher
323 variability in the estimated recognition capabilities of our Ab has been observed when using
324 custom designed NHS-wells with a within-assay precision equals of 10.3% for that custom
325 designed wells batch, in comparison with conventional ones (within-assay precision is less than
326 7.1%; Coussot *et al.*, 2018b). The effects of transportation are evaluated by confronting activity
327 of ground controls stored in DLR with that of CNES ground samples. Remaining activity of
328 both CNES and DLR grounds controls, are not statistically different (Student's *t*-test). No
329 significant effect is also observed with the ground sample named DLR ΔT+UV, which
330 combines long time storage, varying thermal environment mimicking ISS ones, and UV
331 radiations. To complement these data, we run here additional ground thermal cycling
332 experiments to evaluate whether an effect due to a long-term exposure to temperature variations
333 during the mission can be invoked. Ground thermal cycling experiments were carried out on
334 the anti-HRP Ab over a 80°C short peak of temperature, a long cycle mimicking thermal
335 variations of flight samples (see ***section II.6*** of the experimental part), and compared with an
336 identical storage time at 4°C. All assays were performed with eight replicates. Ab activities are
337 presented in **Figure 3**. A degradation of 1/3 of initially active grafted Ab (corresponding to
338 66% of surface active Ab) is shown during the freeze-drying process by comparing data
339 obtained with fresh G Ab that did not undergo freeze-drying with that of rehydrated sample
340 upon opening of the aluminium holder (**Figure 3,** first two shaded bars with grey lines). In
341 addition, comparing results from the overall process, cumulating the freeze-drying step and

342 storage, shaded grey bars indicate a slight but significant difference in activity between the three
343 stored freeze-dried samples (55.1±4.1 for the 4°C storage; 48.5±3.2 for the 80°C peak; 46.7±4.1
344 during the long cycle) and the freeze-dried sample analysed with no storage (66.8±6.7) whereas
345 the percent Ab activity values after the sole freeze-drying process are 83.1±14.5, 69.8±13.1,
346 and 72.5±12.1 for the freeze dried 4ºC storage, the 80ºC peak, and long cycle, respectively,
347 indicating that there were not significant differences ($p>0.05$) between the reference sample
348 with no storage and the 3 stored ones (**Figure 3**, filled grey bars**)**.

349

350 ### *III.2. Resistance of anti-HRP Ab in free format after exposure outside the ISS*

351 Since a 30% functional activity difference in freeze-dried Ab, between the free format and the
352 covalently immobilized one, has been reported in literature depending on the freeze-drying
353 formulation components (Coussot *et al.,* 2018c), and that only free Ab format has been studied
354 in previous experiments on the BIOPAN-6 low Earth orbit platform (Derveni *et al.,* 2012;
355 Derveni *et al.,* 2013), the resistance of free anti-HRP Ab after exposure to real space conditions
356 is detailed hereafter.
357 Previous data using the same freeze-drying solution adjusted to pH 7.4 for Ab immobilization
358 to the NHS-wells showed that free Ab activity losses under 25±2% could not be quantified with
359 the A2HRP method due to sample preparation, multi-step procedure, and handlings (Coussot
360 *et al.,* 2018c). However, the A2HRP method has the advantage of providing reliable and precise
361 data for stability studies of the free anti-HRP Ab, by comparing with conventional competitive
362 inhibition assays. Indeed, changes in Ab properties upon degrading conditions were proved to
363 dramatically affect the data in conventional immunoassays with high variability of the results.
364 It is also restricted to competitive mechanism of inhibition; if other mechanisms occurred at the
365 same time in the sample it would generate uninterpretable data. All the results from the

366 EXPOSE-R2 mission samples illustrated in **Figure 4,** were obtained with the A2HRP method.
367 No significant alteration of the free anti-HRP Ab is observed after both 80°C peak and long
368 cycle exposures (data from ground experiments not shown). As shown by Coussot *et al.* 2018c,
369 a possible additional air moisture degrading effect might have occurred during the storage of
370 the freeze-dried F anti-HRP Ab at 4°C (sealed bag not perfectly hermetic). Owing to the
371 inherent conditions of the rehydration step, this result is not surprising and correlates well with
372 the above observations on the freeze-dried G Ab, and that of free GR stored in DLR
373 (73.7±10.3%). Consequently, considering all the results presented in **Figure 4**, no significant
374 differences (p>0.05) are observed in the binding activity of the anti-HRP Ab, under its free
375 format, with the same order of activity for the flight samples as for the ground ones. As before,
376 a 37% drop in remaining free Ab activity is necessary to detect alterations in Ab binding
377 efficiency from cosmic radiations during flight samples exposure outside the ISS.
378

379 **IV. Discussion**

380 In this study, we evaluated the effect due to a long-term exposure to temperature variations on
381 the activity of anti-HRP Ab in its grafted format but independently from flight samples (**Figure**
382 **3**). Indeed, the effect of short exposure of covalently grafted Ab at elevated temperatures was
383 previously published (Coussot *et al.*, 2018c). It revealed that about 60% of the initial activity
384 of covalently immobilized Ab was preserved during short temperature stress. We reported also
385 that freeze-dried samples suffered less from stress due to temperature exposure than from the
386 rehydration step, probably due to changes in composition of the solid form upon contact with
387 air moisture, which was supposed to lead to only a partial refolding of the freeze-dried Ab.
388 Thus, after exposure of grafted Ab to thermal variations, activity changes, if they occurred,
389 might not be due to an effect of temperature but rather from storage time. Indeed, the results of

390 remaining Ab activity after the freeze-drying process show no discrepancy ($p>0.05$) between
391 the reference sample with no storage and the 3 stored ones (**Figure 3**, filled grey bars). This
392 means that an alteration of Ab bindings other than those occurring during the freeze-drying
393 process/rehydration or thermal variations might be evoked only if there is more than 40% drop
394 in remaining Ab activity (from freeze-dried control) after a long-duration experiment. This is
395 consistent with tests done on GR samples illustrated in **Figure 2**.

396 Measurements performed with passive dosimeters during the EXPOSE-R2 mission revealed, a
397 posteriori, that the absorbed dose difference between cells at upper and lower levels was low
398 (about 3 mGy for the whole mission corresponding to 1.4% of the total). We explained this
399 difference due to an estimated shielding of TLDs of 0,82 g/cm^2 on the upper level (Coussot *et*
400 *al.*, 2018d) so the absorbed dose remains quasi uniform between the two levels. This can explain
401 why the results we obtained for the "upper tray" and the "lower tray" samples have no statistical
402 differences. Indeed, we obtained a remaining activity of 50.6±9.0% for the flight "upper tray"
403 samples, and 62.9±11.0% for the flight "lower tray" for grafted Ab (**Figure 2**). For free format,
404 our results showed that a 37% drop in remaining free Ab activity is necessary to detect
405 alterations in Ab binding efficiency. In other words, this shows that much more than 63% of
406 the free anti-HRP Ab are still functional after their long-duration exposure outside the ISS
407 (**Figure 4**). Consequently, based on all the above considerations, our results show that grafted
408 and free Ab partially keep their recognition capabilities during the overall flight mission, and
409 that the degradations from cosmic radiations, if any, are too small to be detected by our analysis
410 protocols.

411 Nevertheless, due to the high number of potentially hazardous factors encountered during a
412 space mission (McKenna-Lawlor *et al.*, 2012), our experiment demonstrates with confidence,

413 for the first time, that much more than 40% of Ab (whatever the format) survived to the long-
414 duration exposure outside the ISS and remained functional.

415

416 Antibody-based biochips have not been used yet for planetary exploration missions although
417 their high potential for searching tracers of extinct or extant life (Parro *et al*. 2011c). One major
418 concern for an instrument based on this technology is radiation effect issues on the antibodies.
419 Many laboratory experiments have been performed so far at different energies with different
420 particles and high fluences (much more important than suspected for a mission to Mars for
421 instance) to test the ability of antibodies to recognize their target after radiation exposure (Le
422 Postollec *et al.*, 2009a; Le Postollec *et al.*, 2009b; Baqué *et al.*, 2011; de Diego *et al.*, 2011;
423 Baqué *et al*., 2017; Coussot *et al.*, 2017). A 12 days mission on the BIOPAN-6 low-earth orbit
424 platform has been performed to study the effects of cumulative irradiations on two antibodies'
425 ability to bind to their respective antigens (Derveni *et al.*, 2012; Derveni *et al.*, 2013). In the
426 present paper, we improved previous studies to better test the effects of real-space constraints
427 on antibodies during an EXPOSE-R2 mission outside the International Space Station. In terms
428 of radiation, the total accumulated radiation dose recorded by the dosimeters during the
429 EXPOSE-R2 is 220 mGy, which is much higher than those measured during the BIOPAN-6
430 low Earth orbit experiment (2.4 mGy) (Derveni *et al.*, 2012), and is in agreement with the
431 absorbed dose expected during a mission to Mars (Hassler *et al.* 2014, Le Postollec *et al.*
432 2009a). All these studies show two major results: (1) the preparation procedure and analytical
433 steps have to be controlled and validated with care to obtain reliable results. (2) no clear
434 deleterious effect have been reported so far on the antibody and antigen recognition step, in the
435 limit of detection of the analysis protocols.

436 As a consequence, to our opinion, radiation effects on the antibodies should not been considered
437 anymore as an issue for antibody-based instruments dedicated to a planetary mission (in
438 particular for the exploration of Mars).

439

440 **IV. Conclusions**

441 The aim of our study was to test whether space flight conditions might have influences on the
442 performances of Ab-based biochips. A direct, precise and reliable assay was used to evaluate
443 the remaining activity of ground controls and flight samples that underwent long-term storage,
444 temperature variations, and shocks all along the EXPOSE-R2 mission. The results presented in
445 this paper show that cosmic radiation has no significant effect on the antibody recognition
446 ability, independent of the exposed format, *free* or *immobilized onto a solid surface*. These
447 results are in agreement with all the previous ground-based experiments performed on
448 irradiation facilities with different particles at various energies.
449 In that experiment, since we precisely managed all the steps of the analytical protocol and of
450 the sample conditioning under controlled atmosphere, and with all the necessary steps to
451 consider during the overall mission (**Figure 1**), we clearly demonstrated the relevance and
452 adequacy of antibody based instruments to be used for future planetary exploration
453 experiments.

454

455

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461 the analysis of samples.

462

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608

609 **Figure captions**

610 **FIG.1.** An overall view of the EXPOSE-R2 mission with two formats of freeze-dried anti-HRP
611 antibody (Ab): first is grafted format (G) with Ab covalently immobilized on the well-surface
612 and second is free format (F) with Ab prepared *in-solution.*
613 ***1A*) **Pre-flight samples preparation with 1) Freeze-drying procedure, conditioning and screwing
614 of the CNES cells under controlled atmosphere, in Montpellier; 2) Soldering of the CNES cells,
615 integration of CNES cells into the trays, in Paris. Ground references (GR) were stored in CNES
616 (Toulouse) and in DLR (Germany); 3) 56P launch from the cosmodrome in Baikonur,
617 Kazakhstan.
618 ***1B*)** EXPOSE-R2 flight with 4) Integration of the trays into the EXPOSE-R monoblock, 26
619 days stay inside ISS; 5) EVA-39, exposition outside the ISS; 6) EVA-40, removal of the sun
620 shield; 7) EVA-42, end of exposure, return into the ISS and de-integration of the trays; 8) Return
621 to ground, expedition 46 landing, 44S Soyuz capsule.
622 ***1C*)** Post-flight experiments with 9) Disassembly of the PSS carriers, and de-integration of the
623 exposed cells. Ground references (21 GR in DLR and 6 GR in CNES that represent 14F + 13G)
624 brought back to Montpellier; 10) Desoldering of the cells in Montpellier; 11) Analysis of freeze
625 dried samples according to A2HRP method.
626 ***1D)*** A2HRP method principle. A decrease in the HRP-Ab binding events results in a decrease
627 of the absorbance signal (*see* experimental part for details).

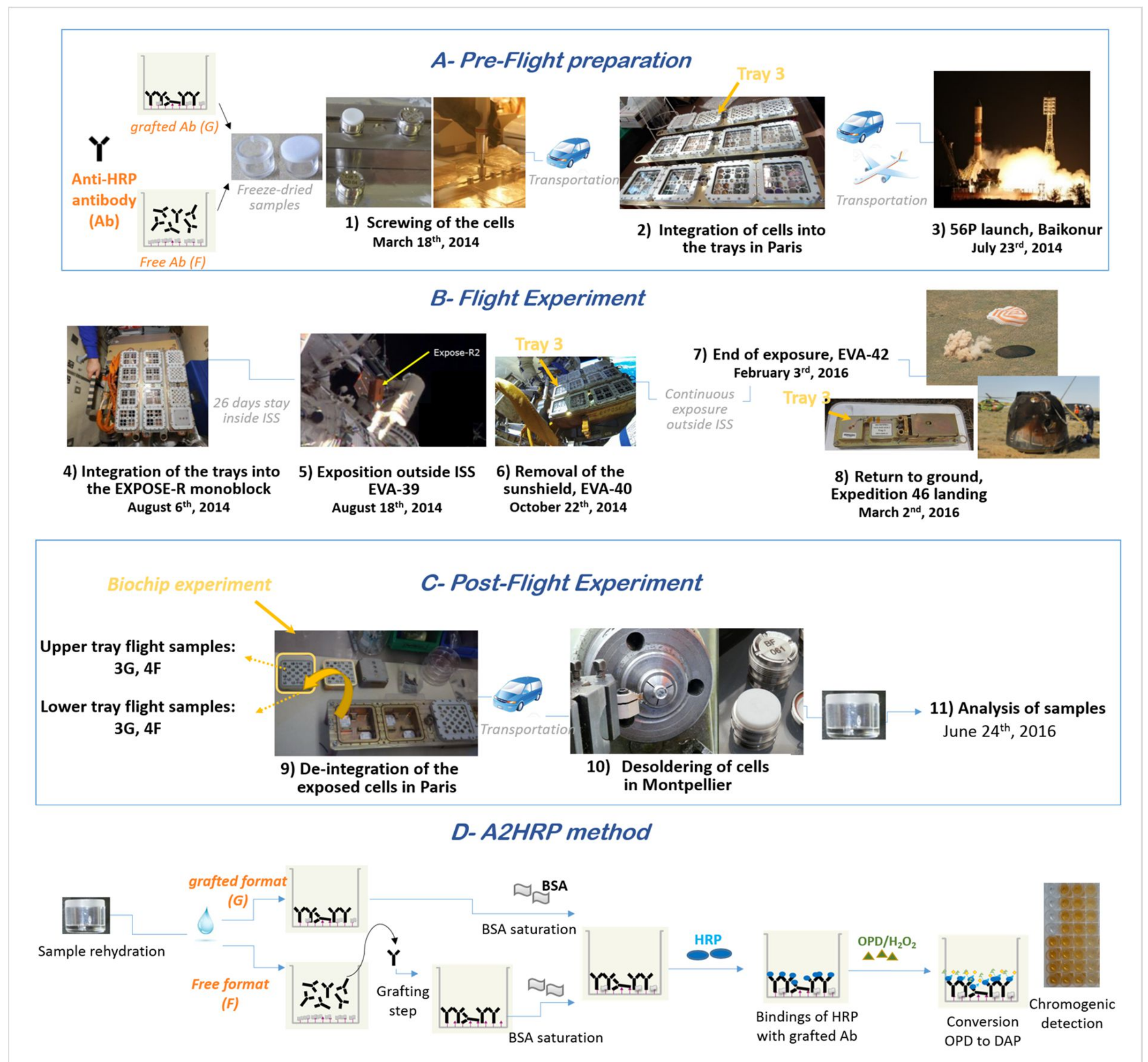
628 **FIG.2.** Binding activity of the grafted anti-HRP Ab obtained for ground controls (number of
629 replicates: CNES *n*= 3; DLR *n*= 2; DLR ΔT *n*=4; DLR ΔT+UV *n*=3) and flight samples (*n*= 3
630 replicates for both lower and upper tray) by the A2HRP method. Each filled grey bar represents
631 the mean and standard deviation (SD) of the Ab activity (normalized value in %, calculated
632 against freeze-dried ground controls stored in DLR at 5°C (*n*=2 replicates)).

633 **FIG.3.** Binding activity of the grafted anti-HRP Ab (G Ab) obtained without and after freeze-
634 drying, without storage (instantaneous rehydration after freeze-drying), with storage at 4°C for
635 freeze-dried control, with sharp rise in temperature (80°C peak), and a thermal cycling
636 experiment mimicking thermal cells exposure outside the ISS (long cycle ΔT) but in accelerated
637 time: one hour of EXPOSE-R2 mission was converted into one minute to carry out this
638 experiment in the lab with similar temperature amplitude (for details see Coussot *et al.*, 2018d).
639 Each bar represents the mean and standard deviation (SD) of the Ab activity (*n*= 8 replicates,
640 normalized value in %), calculated against freshly prepared Ab surface (shaded bars with grey
641 lines) to evaluate cumulative effects or calculated against freeze-dried Ab without storage
642 (filled grey bars) to evaluate the effects of storage and temperature.

643 **FIG.4.** Binding activity of the free anti-HRP Ab obtained for ground controls (number of
644 replicates: CNES *n*= 3; DLR *n*= 3; DLR ΔT *n*=4; DLR ΔT+UV *n*=4) and flight samples (*n*= 3
645 replicates for both lower and upper tray) by the A2HRP method. Each dark bar represents the
646 mean and standard deviation (SD) of the activity (normalized value in %, calculated against a
647 freshly prepared freeze-dried ground reference).

648

649 **FIG.1.**



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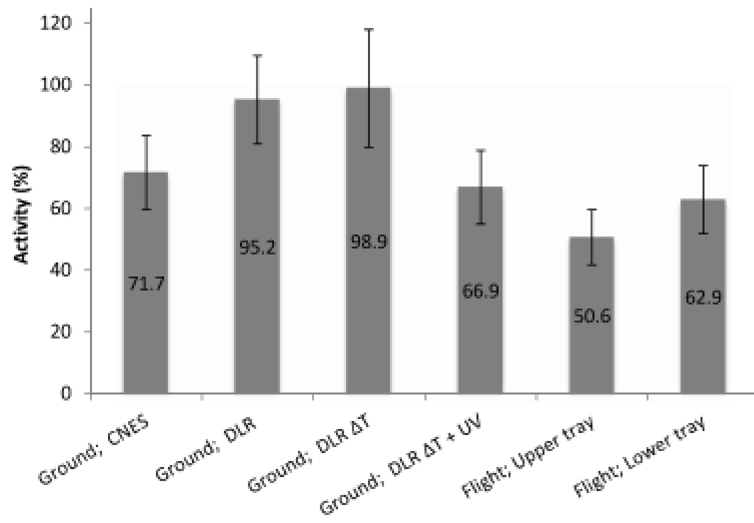
653 **FIG.2.**



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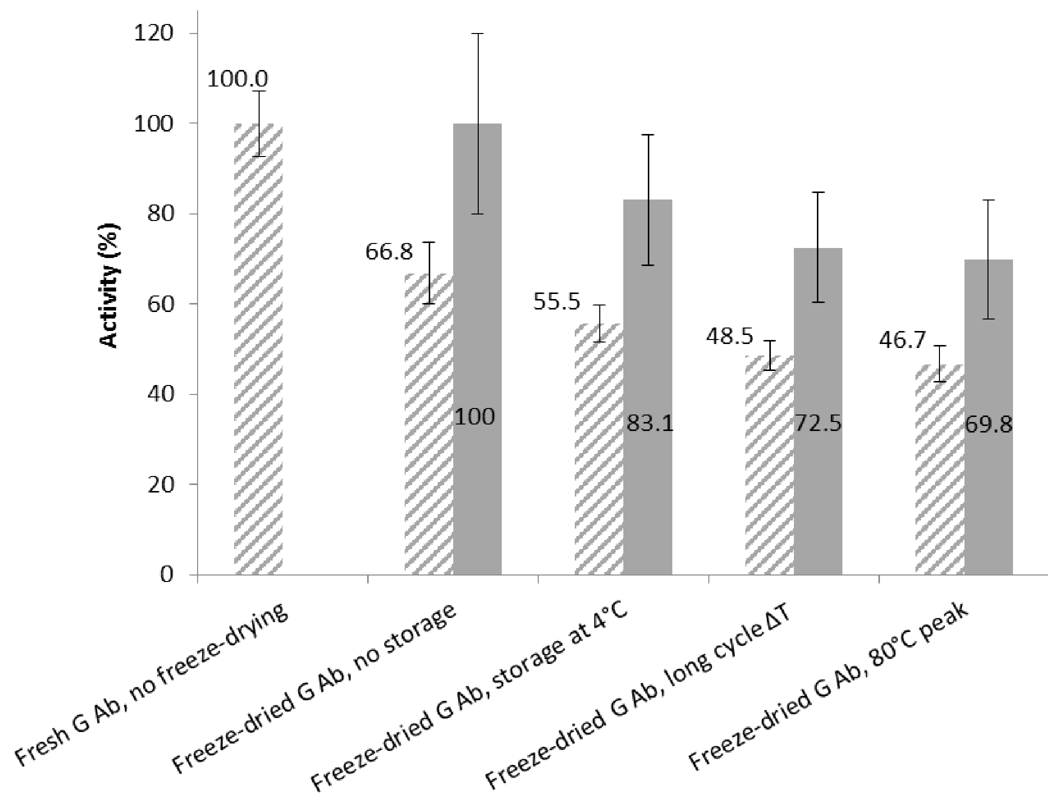
655 **FIG.3.**



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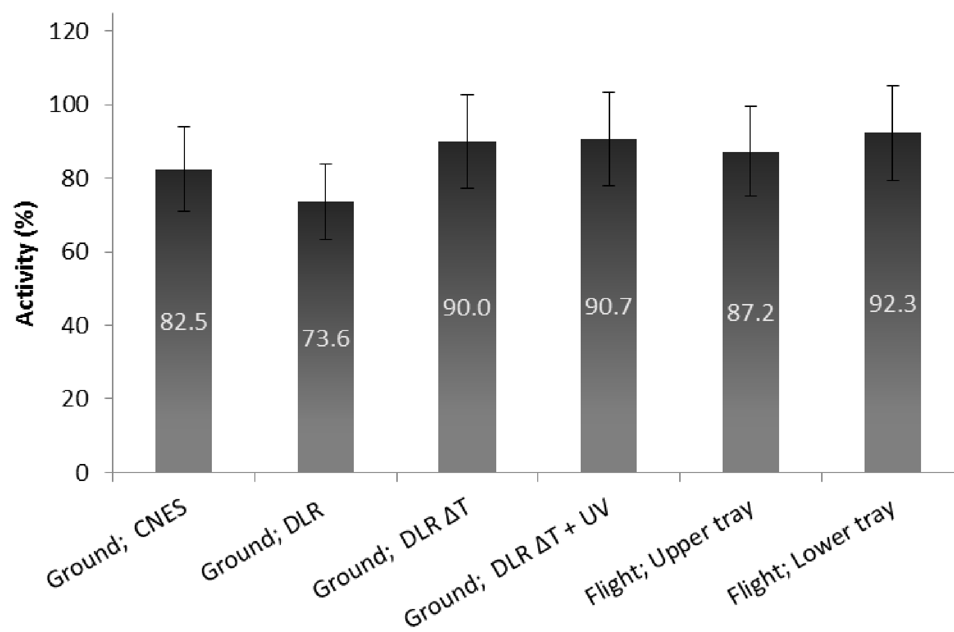
659 **FIG.4.**

660



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