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The IR-RF alpha-efficiency of K-feldspar

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1 **Antibacterial, anti-biofilm activity and mechanism of action of Pancreatin doped Zinc**
2 **oxide nanoparticles against methicillin resistant *Staphylococcus aureus*.**

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9 **Highlights:**

- 10 • Pancreatin coated Zinc oxide nanoparticles (ZnNPs-PK) were prepared and
11 characterized
- 12 • ZnNPs-PK shows bactericidal, anti-biofilms anti-motility and anti-virulence activity
13 against methicillin resistant *Staphylococcus aureus* (MRSA)
- 14 • ZnNPs-PK treatment makes MRSA more sensitive to vancomycin.
- 15 • ZnNPs-PK targets cell membrane and induced ROS generation as mode of action
16 against MRSA.
- 17 • ZnNPs-PK was found to be nontoxic and shows selective antibacterial action.

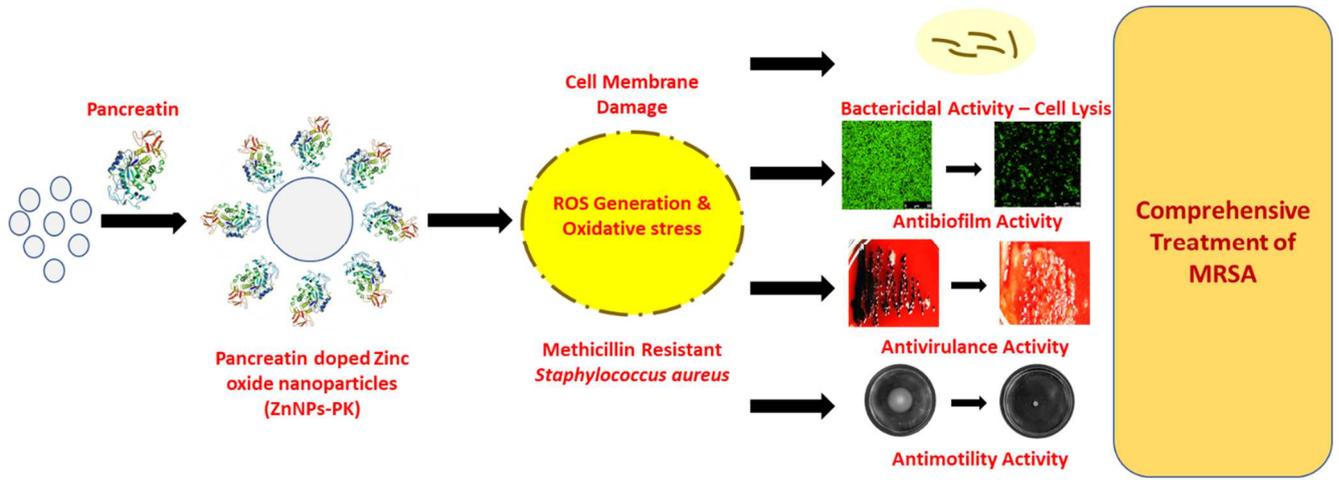
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22 **Graphical Abstract:**



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35 **Abstract**

36 *Staphylococcus aureus* are known to cause diseases from normal skin wound to life
37 intimidating infections. Among the drug resistant strain, management of methicillin resistant
38 *Staphylococcus aureus* (MRSA) is very difficult by using conventional antibiotic treatment.
39 In present study we show that functionalization of zinc-nanoparticles with pancreatin enzyme
40 have anti-bacterial, anti-biofilms, anti-motility and anti-virulence properties against MRSA.
41 Application of the produced nano-composites as treatment on infected swine dermis
42 predominantly reflects the potential treatment property of it. The vancomycin sensitivity of
43 MRSA was significantly increased on application with ZnNPs-PK. Further study revealed
44 ZnNPs-PK targets bacterial cell membrane and induced oxidative damage as its biocidal
45 mode of action. The produced nanoparticles were found completely non-toxic to human's
46 keratinocytes at its bactericidal concentration. Overall, this study emphasizes the potential
47 mechanisms underlying the selective bactericidal properties of Pancreatin doped zinc oxide
48 nanoparticles against MRSA. This novel nanoparticle strategy may provide the ideal solution
49 for comprehensive management of MRSA and its associated diseases with minimising the
50 use of antibiotics.

51 **Keywords:** Zinc-oxide-Nanoparticles; pancreatin; MRSA; Selective-bactericidal; Anti-
52 biofilms;

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58 **1. Introduction**

59 *Staphylococcus aureus* is a normal micro flora present in humans and results into an
60 opportunistic pathogen with respect to some immuno-suppressive host circumstances[1]. *S.*
61 *aureus* infections include extremities from normal skin wound to life intimidating infections
62 like pneumonia, and exotoxins syndromes, endocarditis, and septicaemia[1]. Extended and
63 abandoned usage of bacteriostatic or bactericidal antibiotics has also resulted in the
64 emergence of multidrug resistance strains of *S. aureus* (MDR)[2]. Amongst the drug resistant
65 strains, *methicillin resistant Staphylococcus aureus* (*MRSA*) is an exemplar of third
66 generation antibiotic defiant bacteria. The presence of the *mecA* gene in the staphylococcal
67 cassette chromosome *mec* is responsible for the methicillin resistant. *MRSA* could cause
68 broader infection across communities inside and outside of the hospital[3]. *MRSA* are found
69 to be more resistant to antimicrobial agent than other nosocomial pathogens[4]. Therefore, it
70 becomes tough to be relieved from the infection associated with *MRSA* using conventional
71 antibiotic treatment. This could be a reason why infections associated with *MRSA* have
72 reached pandemic extent worldwide[5].

73 The existence of various virulence factors such as slime production, exopolysaccharide
74 production, gliding motility, staphyloxanthin pigment production, etc. in *S. aureus* might
75 enhance their probable intimidation particularly that majority of them have enhanced
76 pathogenicity with multi-drug refusal, results it complicated to treat[6]. The most crucial
77 virulence properties of bacteria are the biofilms formation. Biofilms have immense
78 significance for civic wellbeing because of their significant role in spreading of certain
79 contagious diseases[7]. Biofilms associated bacteria are considerably more adaptable to
80 ecological stresses or toxic substances like antibiotics and biocides, than planktonic cells[8].
81 Therefore, for comprehensive treatment of *MRSA* associated disease, it is important that
82 antibacterial agent should have anti-biofilms and anti virulant properties as well.

83 Recently, Metal nanoparticles are emerged as a new weapon to combat different bacterial
84 diseases [9]. According to the different metal nanoparticles, Zinc oxide (ZnO) nanoparticles
85 are reported to low cost, bio-safe and much less toxic for human use [10]. Zinc oxide
86 nanoparticles are reported to have broad spectrum antimicrobial activities even act against
87 different drug resistant strains of pathogenic bacteria including *MRSA* [11]. Therefore, zinc
88 oxide nanoparticles may consider as an alternative to conventional antibiotics for the
89 treatment of diseases caused by *MRSA*.

90 On the other hand, enzymes like Pancreatin also play as great anti-microbial agents[12].
91 Pancreatin made up of combination of three enzymes – amylase, protease and lipase. It has
92 been reported earlier that, individually these enzymes eliminate biofilms of *MRSA*[13]–[15].
93 In addition, this may be a reason for anti-biofilms activity of pancreatin against *MRSA*.

94 Here in this study, we may hypothesize that ZnO nanoparticles and pancreatin should be a
95 great combination for comprehensive management of *MRSA* associated diseases. For this
96 ZnO nanoparticles were daubed with pancreatin enzymes (ZnNPs-PKs).

97 Our main objective of this study is to produce a pancreatin daubed zinc oxide nano-
98 composites which produce less toxicity but superior anti-bacterial, anti-biofilm and anti-
99 virulent activity against *MRSA*. To achieve that, in this study we first prepared and
100 characterized ZnNPs-PKs and then investigated how ZnNPs-PKs (I) inhibits growth, (II)
101 affects biofilm formation and other virulence factors of *MRSA*[16].

102 **2. Materials and methods:**

103 **2.1. Micro-organisms:**

104 Clinically isolated culture of methicillin resistant *Staphylococcus aureus* collected from
105 Department of Medical Microbiology, Nil Ratan Sircar Medical College & Hospital, Kolkata,
106 West Bengal, India and maintained in Department of Microbiology, Techno India University,
107 West Bengal was used by growing the bacterial culture on Luria agar, and sub-cultured on
108 Luria Broth prior to each experiment by adjusting turbidity to 0.5 O.D.

109 **2.2. Chemicals and compositions:**

110 Analytical grade zinc nitrate $Zn(NO_3)_2 \cdot 6H_2O$; (Sigma-Aldrich-14436) and potassium
111 hydroxide (KOH) (Merck's global life science, Millipore Corporation, USA). Hydrazine
112 hydrate (N_2H_4 ; 98.0%) of Sigma-Aldrich (St Louis, MO, USA). Crystal violet solution (99%)
113 of (Merck Merck's global life science, Millipore Corporation, USA) which was used to stain
114 biofilms. 96-wells plates and 12-wells plates (Tarsons Products (P) Ltd.) were used of
115 biofilms inhibition assay.

116 **2.3. Synthesis and characterization of nano-composites:**

117 Synthesis of Zinc oxide nanoparticles were prepared by following established protocol [17].
118 At first, Zinc oxide nanoparticles (ZnNPs) were prepared by precipitation method. Potassium
119 hydroxide solutions (KOH) (0.4M) were slowly added with Zinc nitrate (0.2M) in deionised
120 water at room temperature. The solution was mixed by vigorously stirring until it formed
121 turbid white suspension. The produced white milky suspension was centrifuged at 5000 rpm
122 for 20 minutes and washed thrice with deionised water. Pellet was further washed with
123 ethanol. The resultant product was then calcined in air atmosphere at 500°C for 3 hr. For the
124 preparation of pancreatin daubed zinc oxide nanoparticles (ZnNPs-PK), 2mg/ml pancreatin

125 enzyme solution was added to the 10mg/ml zinc oxide nanoparticles solution. This was then
126 stirred vigorously under room temperature for 2hrs. After that suspension was centrifuged at
127 5000 rpm for 20 minutes and washed thrice with deionised water. The prepared nano-
128 composites were the characterized by following way.

129 To observe the optical property of prepared nanocomposites, samples were analysed for UV–
130 vis spectroscopic studies (UV-visible Spectrophotometer 2206, SYSTRONICS) at room
131 temperature operated at a resolution of 1 nm between 200 to800 nm ranges Dynamic Light
132 Scattering (DLS) analysis was done with a Zeta sizer Nano ZS (Malvern Instruments)
133 according to standard method with some modifications. The amount of enzyme adsorbed on
134 the ZnNPs-PK surface was evaluated by Bradford assay using UV-VIS spectrophotometer.

135 **2.4. Antibacterial activity of ZnNPs-PK**

136 **2.4.1 Determination of minimal inhibitory concentration (MIC) and Minimum** 137 **bactericidal concentration (MBC):**

138 Determination of minimal inhibitory concentrations (MICs) of ZnNPs and ZnNPs-PK for
139 *MRSA* was determined according to standard protocol |The bacterial suspension were treated
140 with or without different concentration of ZnNPs-PKs and were incubated at 37°C overnight
141 under shaking condition. After incubation, the MIC values were obtained by checking the
142 turbidity of the bacterial growth. The MIC value corresponded to the concentration that
143 inhibited 99% of bacterial growth[18].

144 Minimum bactericidal concentration (MBC) was estimated by spreading plating from MIC
145 assay and incubated overnight. The lowest concentration of the nanoparticles which
146 completely killed the tested bacteria was observed and tabulated as MBC value[18].

147 **2.4.2 Death kinetics:**

148 To determine the death rate of *MRSA* in presence of ZnNPs-PK, an overnight culture was
149 diluted 100 times in fresh LB and allowed to cultivate at 37°C under shaking condition upto
150 log phase ($\sim 5 \times 10^7$ cells/ml). ZnNPs-PK was supplemented at MBC and the cells were
151 incubated overnight at 37°C under shaking condition. At different time interval of 60 mins,
152 cell aliquots were withdrawn, serially diluted and spread on agar plates to evaluate quantity
153 of viable cells[19][20]. According to the slope of the plot (Log N_t/N_0 against time associated
154 with incubation), the death rate of ZnNPs-PK treated cells was evaluated[18].

155 **2.5 Anti-biofilms activity of ZnNPs-PK**

156 **2.5.1 Initial biofilms inhibition:**

157 In brief, overnight cultures of *MRSA* were inoculated into wells of a polystyrene 24-well cell
158 culture plate with LB containing various concentrations of ZnNPs-PK and then incubated at
159 37°C for 24h without agitation. After incubation, LB was removed, and the wells were
160 thoroughly washed with sterile phosphate-buffered saline (PBS) to remove planktonic and
161 non-adherent cells.

162 For determination of biofilm biomass sessile cells were stained with 0.05% crystal violet, the
163 excess of which was then rinsed off using distilled water. After dissolution with 95% ethanol,
164 the biofilm biomass was determined by reading OD₅₉₅. The percentage biomass formation
165 was determined using the following equation.

166 Percentage Biofilm Formation = $\left[\frac{\text{Test Sample OD}_{595} \text{ nm}}{\text{Control sample OD}_{595} \text{ nm}} \right]$
167 $\times 100$].

168 To investigate cell viability, adherent bacteria in each well were resuspended by vigorous
169 pipetting and vortexing followed by sonication and then were serially diluted 10^6 - through
170 10^8 -fold and then plated onto LA plates. The agar plates were incubated at 37°C for 24 h
171 before bacterial colonies were counted.

172 **2.5.2 Mature biofilms degradation:**

173 Biofilms were cultivated in LB broth in 24-well polystyrene plates at 37°C without shaking.
174 After 24hrsincubation, the broth were removed and the biofilms were rinsed with PBS and
175 then supplemented with fresh LB broth and ZnNPs-PK with different concentrations[20].
176 After another 24-h cultivation, the formed biofilms were washed with PBS and stained using
177 crystal violet, solubilized with ethanol, and eventually quantified at 595 nm using a
178 microplate reader.
179 For cell viability, sessile cells were washed with PBS, then resuspended by vigorous pipetting
180 and vortexing and then sonicated for 30 s. The number of CFU/biofilm was quantified by LB
181 agar plating.

182

183 **2.5.3 Microscopic observation of biofilm:**

184 **2.5.3.1 Light Microscopy**

185 For light microscopy, the biofilm assay was performed with small glass slides (1×1cm)
186 placed in the wells of the 24-well polystyrene plate. ZnNPs-PK was added at its MIC to the
187 preformed biofilms incubated at 37°C for 24 h. After incubation, planktonic cells were
188 removed, and the biofilm formed on the glass slides was stained using crystal violet dye for 5
189 min. It was then gently washed with PBS and allowed to dry for 5 min. Then, the slides were
190 viewed under a light microscope at 40x magnification and images were taken using digital
191 camera[21].

192 **2.5.3.2 Confocal Microscopy**

193 The surface topology of *MRSA* biofilm architecture were visualized under Confocal Laser
194 Scanning Microscope (CLSM) by forming biofilm on glass slide placed on 24-well

195 polystyrene microtiter plate[20]. To determine the effect of ZnNPs-PK to disrupt the mature
196 biofilms, MIC of ZnNPs-PK was added to the mature biofilms and incubated overnight at
197 37°C, then the glass slides were taken and washed with PBS followed by staining with
198 acridine orange and observed using CLSM (Carl Zeiss LSM700, Jena, Germany)[21].

199 **2.6. Anti-motility assay:**

200 **2.6.1 Sliding motility:**

201 The spreading ability of MRSA on soft LB agar was assessed in the presence and absence of
202 ZnNPs-PK. LB soft agar was prepared with 24 g/lit of agar and poured into the petri dish
203 plate. Overnight, treated (MIC concentration) and un-treated cultures of MRSA were spotted
204 in the middle of the plate and air dry for 20 mins at room temperature and incubated for 48
205 hours at 37°C. Sliding motility of selected strains was estimated through measuring the
206 expansion of colony growth from the inoculation point[21].

207 **2.6.2 Colony spreading assay:**

208 To determine the anti-colony-spreading activity of ZnNPs-PK 5 ml of LB agar (0.4%)
209 medium with MIC of ZnNPs-PK and without ZnNPs-PK was prepared and then poured over
210 the LB agar plates (2%). After solidification, MRSA were spotted on the centre of the plate
211 and incubated for 24hrs at 37 °C. Further, the diameter of the colony was measured to
212 determine the effect of ZnNPs-PK on spreading[21].

213 **2.7 Anti-virulence study:**

214 **2.7.1 Slime production:**

215 The ability ZnNPs-PK to reduce slime synthesis of MRSA was determined by Congo red agar
216 (CRA) analysis. ZnNPs-PK at its MIC was streaked to CRA and was incubated at 37°C for

217 24 hours. After incubation the changes from black colonies to Bordeaux red colonies was
218 noted. The process was practiced in triplicates[22].

219 **2.7.2 Exopolysaccharide production:**

220 To determine EPS production, *MRSA* culture with and without MIC of ZnNPs-PK was grown
221 on LB media in 24 well plate for 24 h at $28 \pm 2^\circ\text{C}$. After incubation, the non-adherent cells
222 were aspirated and removed and 0.5% NaCl was added. These cells suspended in 0.5 % NaCl
223 were transferred to fresh sterile test tubes and added with equal volume of 5% phenol. To that
224 solution, 5 volume of concentrated sulfuric acid containing 0.2% hydrazine sulphate was
225 added and incubated in dark for 1 h and the absorbance was measured at 490nm[23].

226 **2.7.3 Staphyloxanthin production assay:**

227 The ability of ZnNPs-PK to reduce the staphyloxanthin pigment secretion was investigated
228 by carotenoid extraction. *MRSA* at its mid log phase were sub-cultured by diluting it to 1:10
229 (V/V) and ended up to an ultimate volume of 5 ml in LB broth. To the culture, ZnNPs-PK at
230 MIC was added and incubated at 37°C for 48 hours under shaking conditions. After
231 incubation the cultures were centrifuged at 10,000rpm for 5 mins at 4°C . Culture devoid of
232 ZnNPs-PK was considered as control. The change in color of the culture from golden yellow
233 was measured at an absorbance of 462 nm using UV-VIS spectrophotometer[24].

234 **2.7.4 Bacterial Hemolysis activity:**

235 Blood samples of 5mL mixed with acid citrate dextrose to avoid clotting. These samples were
236 first centrifuged, removed the supernatant and then diluted with PBS at 1:10 ratio[25].
237 Diluted blood samples were inoculated with un-treated and treated bacterial cultures were
238 added to it[20]. These combinations were incubated overnight at 37°C . Prior to incubation,
239 the tubes were centrifuged at 3,000 rpm for 20 min. Absorbance of supernatants was

240 calculated at 540 nm and the rate of haemolysis was intended in percentage for the triplicate
241 data[22].

242 **2.7.5 Plasma clamping assay:**

243 Overnight cultures of *MRSA* were re-suspended in PBS. Human blood plasma was removed
244 from RBC by centrifugation at 10,000 rpm for 5 minutes. 20 μ L of plasma was given on the
245 surface of glass slides as small circular drops. In addition, un-treated and treated *MRSA*
246 suspension was added to each of the slides having the plasma samples. Blood plasma with
247 un-treated *MRSA* served as positive control, and plasma with application of distilled water is
248 served as a negative control. The suspensions were blended uniformly macroscopic
249 agglutination of bacterial cells was observed in each trice times[26].

250 **2.8 Ex-vivo porcine skin model**

251 Porcine skin majorly includes Swine dermis. Porcine samples were collected from excess
252 trash of local meat shop. The sample was cut in appropriate dimensions (1x1cm) and was
253 surface sterilised with 1% isopropanol for 2hours. The sample skins were place aseptically in
254 sterile stainless-steel clamps having two to three holes for inoculations. Treated and untreated
255 bacterial samples with or without ZnNP-PK were inoculated in the holes of the clamps at
256 MIC and sub-MIC concentrations. Samples treated with sterile media broth were served as
257 negative controls and samples applied with untreated *MRSA* were served as positive or
258 infective controls. The setups were incubated for 48-72 hours at 37°C. Each setup was
259 experimented in triplicate.

260 **2.9. Mechanisms of action:**

261 **2.9.1 ROS generation:**

262 ROS generation in nanoparticles treated cells were investigated using flow cytometric
263 technique. The DCFH₂-DA invade the cell of *MRSA* and combines with ROS to build the
264 highly fluorescent composite 2,7-dichlorofluorescein. After treatment with nanoparticles,
265 *MRSA* were cultured and flashed thoroughly with PBS. The cell pellet was taken and a
266 homogenous suspension up to 1mL was prepared by PBS buffer[27]. Subsequently, the cells
267 were incubated with 1.5 mL of 100 μM DCFH₂-DA for 30 min at a temperature of 37°C. The
268 ROS production was evaluated by flow cytometry (Model: FACSV_{er}se flow cytometer,
269 Becton Dickinson). Data were evaluated by FCSE_xpress Software.

270 **2.9.2 Membrane potential change:**

271 Membrane depolarisation was measured by Rhodamine-123 (Rh123) dye. After treatment
272 with nanoparticles, *MRSA* were cultured and flashed thoroughly with PBS. The cell pellet
273 was taken and a homogenous suspension up to 1mL was prepared by PBS buffer. Rh123
274 reagent was added and incubated in the dark for 10 minutes and then analysed by flow
275 cytometry (Model: FACSV_{er}se flow cytometer, Becton Dickinson). Data analysed by
276 FCSE_xpress Software.

277 **2.9.3 Membrane Damage:**

278 Propidium Iodide (PI) can enter the bacterial cell membrane only when it has been
279 permeabilized through an agent, and bind with DNA and gives fluorescence. The
280 fluorescence emission can be detected by flow cytometer. After treatment the cells were
281 washed in PBS buffer and incubated with PI (1.3 μg/ml) at 37°C for 20 min in dark. The PI
282 fluorescence was measured in the flow cytometer (Becton Dickinson (BD) FACSV_{er}se).
283 Data analysed by FCSE_xpress Software.

284 **2.10 Combination study of ZnNPs-PK with vancomycin against MRSA**

285 Combination activity of ZnNPs-PK combined with commercial drugs of vancomycin was
286 evaluated against MRSA. For this we first determined the MIC of vancomycin against
287 MRSA. Then MRSA were treated with $\frac{1}{4}$ and $\frac{1}{2}$ MIC concentration of ZnNPs-PK and
288 vancomycin individually and in combination. The inhibitory activity was determined by
289 checking the turbidity of the bacterial growth. This was measured by spectrophotometrically
290 at a wavelength of 600nm.

291 **2.11 Cytotoxicity study on Keratinocytes**

292 HaCaT cells were seeded in 96-well plates at a density of 10^4 cells per well in 0.2 ml of
293 DMEM: Ham's F12 (1:1 v/v) with 10% FBS and 1% antibiotics and was cultured at 5% CO_2
294 and 37 °C for 24 h [30]. Growth medium in the wells was replaced after 24 h with medium
295 containing different concentration of ZnNPs-PK and incubated for 24 h. The medium was
296 removed thereafter and replaced with 100 μl of medium containing 3-(4, 5-dimethyl-thiazol-2-
297 yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4 h. The unreduced MTT was
298 taken out, and 200 μl of DMSO was added to each well to dissolve the MTT formazan
299 crystals. The content was mixed properly, and absorbance was measured at 595 nm in a
300 microplate reader.

301 **3. Results:**

302 **3.1. Characterisation of nanoparticles:**

303 The absorption spectra of the ZnNPs (Fig.1A) had a broader band with a maximum at 342nm.
304 After doping with pancreatin we also spectrophotometrically characterized the nanoparticles.
305 The absorption spectrum of ZnNPs-PK showed an increased absorption at 280nm indicating
306 the binding of enzyme to the ZnNPs nanoparticles.

307 Average particle size, distribution and polydispersity index (PDI) of synthesized Zn-NPs and
308 ZnNPs-PK in solutions were evaluated by DLS technique. The DLS pattern revealed that
309 ZnNPs had a Z average diameter of 5nm. Which was significantly increased to 45nm
310 (Fig.1B). Hence it may hypothesize that binding of pancreatin to ZnNPs increased the
311 particles size of the nanoparticles. The PDI value of the synthesized ZnNPs was 0.641
312 indicating nearly monodisperse distribution. Little change in the PDI value was observed
313 after the binding with pancreatin, indicating that the size distribution was not affected by the
314 ZnNPs-PK interaction.

315 The percent of protein adsorbed on the functionalized nanoparticles surface was $0.47 \pm 0.4\%$
316 (W/W) as determined by Bradford assay. That indicates binding of few micrograms of
317 proteins per milligram of nanoparticles.

318 **3.2 Antibacterial activity of ZnNPs-PK:**

319 **3.2.1 Determination of minimum inhibitory concentration of ZnNPs and ZnNPs-PK** 320 **against MRSA**

321 At first, we determined comparative antibacterial activity of ZnNPs and ZnNPs-PK against
322 the *MRSA* (Fig.2A). For this, we did broth dilution method to determine the minimum
323 inhibitory concentration of ZnNPs and ZnNPs-PK against the *MRSA*. Different
324 concentrations of the nano-composites were used to treat *MRSA* for the estimation of the MIC
325 value. After incubation, spectrophotometric analysis revealed that the MIC₉₀ for ZnNPs and
326 ZnNPs-PK was 75 and 156 $\mu\text{g/mL}$ respectively. This indicates ZnNPs-PK nanoparticles
327 exhibit greater antimicrobial potential than ZnNPs. Thus ZnNPs-PK nano-composites were
328 used to study further antibacterial, anti-biofilms and anti virulence activities against *MRSA*.

329 To understand the mode of antimicrobial activity, MBC concentration of ZnNPs-PK against
330 *MRSA* was determined. The MBC concentration was found to be 120 μ g/mL. After that, as
331 previous reports suggests[31], we determined the tolerance value to understand the nature of
332 antimicrobial activity of the nano-composites. The tolerance value was 1.6 indicated
333 bactericidal activities of the ZnNPs- PK against *MRSA*.

334 **3.2.2 Death kinetics:**

335 As we found prepared nanoparticles are bactericidal in nature, hence definitely these
336 nanocomposites have an effect on the growth of bacteria and that can be evaluated by
337 investigating the death kinetics of *MRSA* in presence and absence of ZnNPs-PK. For this we
338 treated *MRSA* with MBC concentration of nanoparticles and subsequently the viable cell
339 counts were measured at different times of exposure up to 6 hours, because significant
340 amount cell killing occur within this period. The rate of death percentage of *MRSA* after the
341 treatment was calculated from slopes of straight-line curve (Fig.2B) to be 0.87h⁻¹. In the
342 untreated *MRSA* the growth rate was 0.72h⁻¹. This result strengthened the bactericidal nature
343 of our nano-composites.

344 **3.3. Anti-biofilms activity of ZnNPs-PK against *MRSA***

345 **3.3.1 Initial biofilms inhibition:**

346 At the initial stage of disease progression, planktonic cells are always trying to attach to the
347 substratum or the surface for colonization. That leads to the formation of biofilm. Therefore,
348 inhibition of this initial attachment may be a key factor for finding promising antibiofilm
349 agents. The effects of ZnNPs-PK on inhibition of initial biofilms formation by *MRSA* was
350 determined by enumerate viable sessile cells and crystal violet assay. $\frac{1}{2}$ MIC and MIC
351 concentration of ZnNPs-PK treatment reduced the viable sessile cells by 4 and 7log
352 (CFU/well) respectively as compared with control (Fig.3A). Similarly, the biofilms biomass

353 was also decreased to 50 and 30 % after treatment with MIC and $\frac{1}{2}$ MIC concentration of
354 ZnNPs-PK. (Fig.3A). We also investigated effect of only ZnNPs on initial biofilms formation
355 of *MRSA*. We found at 75 μ g/ml of ZnNPs treatment could not showed any significant
356 antagonistic effect on survivability of sessile cells of *MRSA*.

357 **3.3.2 Preformed biofilms degradation:**

358 As we found an encouraging result about initial biofilm formation inhibition by ZnNPs-PK,
359 we extended our study to understand the disruptive potential of our prepared nanoparticles on
360 preformed biofilms. Prior treatment, we first incubated *MRSA* for 48h to allow the biofilms
361 formation. The $\frac{1}{2}$ MIC and MIC concentrations of ZnNPs-PK treatment resulted in a
362 remarkable reduction of the preformed biofilms in *MRSA* (Fig.3A). Similarly, the sessile cells
363 of preformed biofilms were eradicated by ZnNPs-PK in a concentration-dependent manner
364 (Fig.3B).

365 **3.3.3 Microscopic observation of biofilms inhibition by ZnNPs-PK:**

366 Both the light and confocal microscopic study confirmed disruption of *MRSA* biofilms by
367 ZnNPs-PK. For light microscopic study, application of crystal violet on the mature biofilms
368 by *MRSA*, untreated and treated with ZnNPs-PK at its MIC concentrations. Biofilms
369 disruption was clearly observed as the untreated samples retained more stains than the treated
370 samples (Fig.3B). The consequence of ZnNPs-PK to disturb the mature biofilms of *MRSA*
371 was also examined with CLSM by using acridine orange staining. It is apparent that there is a
372 decrease of green fluorescence in treated samples when compared to that of control indicating
373 disruption of biofilms. All these data suggest ZnNPs-PK effectively eradicate the biofilm
374 formation of *MRSA*.

375 **3.4 Anti-motility activity of ZnNPs-PK against *MRSA***

376 **3.4.1 Sliding assay:**

377 Gram-positive MRSA is a non-flagellated bacteria, hence they can spread by means of sliding
378 motility and that aided to increased biofilm formation on the surface[32]. Thus, to understand
379 the effect of ZnNPs-PK on sliding motility of MRSA was determined by inoculating a culture
380 of MRSA in presence and absence of ZnNPs-PK on the centre of semisolid growth media and
381 incubated for 48 hrs at 37°C. In control, we found the sliding motility was well established,
382 wherein the plates treated with nanoparticles significantly reduced the sliding motility of
383 MRSA (Fig.4A).

384 **3.4.2 Colony spreading assay:**

385 In colony spreading assay, we found untreated control MRSA appeared as rapid and
386 expanded colony spreading on the agar surface, whereas ZnNPs-PK treated MRSA showed a
387 notable reduction in spreading as compared to control (Fig.4A).

388 **3.5 Anti-virulence activity of ZnNPs-PK against MRSA**

389 **3.5.1 Exopolysaccharide and Slime production:**

390 The Slime and exopolysaccharide production by bacteria were considered as key virulent
391 factors for attachment, colonization and biofilm formation within host[33]. Therefore,
392 reduction of both slime and exopolysaccharide production is an important criterion for an
393 anti-biofilm agent. In this study, we evaluated the capability of ZnNPs-PK to reduce the slime
394 and exopolysaccharide production of MRSA was analysed by Congo red agar (CRA) test and
395 EPS quantification analysis respectively. In CRA test ZnNPs-PK at its MIC concentration
396 reduced the slime production which was determined by color alteration from black to
397 bordeaux red (Fig.5A). On the other hand, EPS production was spectrophotometrically

398 quantified in treated and untreated samples. We found EPS production was significantly
399 reduces in MIC treated MRSA as compared to the control (Fig.6A).

400 **3.5.2 Staphyloxanthin production assay:**

401 Staphyloxanthin protects *MRSA* from oxidative stress as well as from the host immune
402 system[34]. Therefore, reduction of staphyloxanthin should be an advantageous for an
403 antimicrobial agent. We found in our study, staphyloxanthin production of *MRSA* was
404 significantly reduced at MIC treatment of ZnNPs-PK as in Fig.6A. Therefore, it can be
405 hypothesized that ZnNPs-PK makes susceptible *MRSA* towards oxidative stress.

406 **3.5.3 Bacterial Hemolysis activity:**

407 *S. aureus* produces a series of haemolysins which lyses erythrocytes and various leukocytes
408 such as neutrophils, monocytes, granulocytes and macrophages[35]. The effectiveness of
409 ZnNPs-PK to reduce the haemolytic virulence property of *MRSA* strains, erythrocytes was
410 exposed to MRSA with or without treated with ZnNPs-PK. We observed that ZnNPs-PK
411 treated *MRSA* showed anti-hemolytic activity. Up to 80% inhibition in haemolytic activity
412 was detected upon treatment with ZnNPs-PK (Fig.6A).

413 **3.5.4 Plasma clumping assay:**

414 Clumping factors are key virulence of *MRSA* for their pathogenicity and survival against host
415 immune system®. In this study, to understand the ability of ZnNPs-PK to inhibit clumping
416 factors of *MRSA*, we evaluated bacterial cell clumping activity using the slide coagulation test
417 with blood plasma. In the absence of nanoparticles, *MRSA* was found to readily agglutinate in
418 blood plasma, whereas in ZnNPs-PK treated *MRSA*, less clumping activity was observed
419 (Fig.5B).

420 **3.6 Ex-vivo porcine skin model to understand the anti-infective nature of ZnNPs-PK**
421 **against MRSA**

422 Porcine skin surface was inoculated with MRSA with or without treatment at MIC of ZnNPs-
423 PK. The skin surface was the cultured 24 h for biofilms formation and physical appearance of
424 infections (Fig.8A). The established bioburden was characterized by plating to determine
425 CFU/ml. After 24h, we noted 9.45×10^4 CFU/cm² in untreated skin surface. However, in MIC
426 of ZnNPs-PK treatment significantly reduced the bioburden of MRSA to 3×10^4 CFU/cm².

427 **3.7. Mechanisms of action of ZnNPs-PK against MRSA**

428 **3.7.1 ZnNPs-PK induced the oxidative stress in MRSA**

429 Oxidative stress levels in MRSA after treatment with ZnNPs-PK was determined by using the
430 2',7'-dichlorofluorescein diacetate (DCFDA) assay. We noted that by flow cytometric assay
431 mean fluorescence intensity of green fluorescence in MRSA treated with MIC of ZNNPs-PK
432 was significantly increased as compared with the untreated cells. This indicates ZNNPs-PK
433 induced oxidative stress by means of ROS generation in MRSA and this may be a possible
434 mechanism responsible for the antibacterial effects of nanoparticles (Fig.6B).

435 **3.7.2 Membrane potential change:**

436 Change in membrane potential after treatment with antimicrobial was considered as another
437 antimicrobial mechanism. Membrane potential has a key role in bacterial physiology[36].
438 Therefore change in membrane potential is an early onset of injury in bacteria. In our study it
439 was evaluated by flow cytometric assay using rhodamine 123. After exposure to ZnNPs-PK
440 at MIC a considerable decrease of MFI values was observed respectively as compared with
441 control (Fig.7A).

442 **3.7.3 Membrane Damage:**

443 Next to understand the membrane damage; we used propidium iodide-based flow cytometric
444 method. In untreated *MRSA*, only a small proportion of cells 5% were stained with PI
445 indicating lesser membrane damage. However, after treatment with MIC of ZnNPs-PK, the
446 bacterial cells with damaged membrane stained by PI were increased to 77% (Fig.7B).
447 Therefore, it can be hypothesized that ZnNPs-PK targets bacterial cell membrane for its
448 antimicrobial activity.

449 **3.8 Combination study of ZnNPs-PK with vancomycin against MRSA**

450 In our study we found the MIC value of vancomycin against MRSA was 1µg/ml. It has been
451 reported that vancomycin MIC against MRSA in the range of 0.5 to 2 µg/ml would have
452 significant clinical implications®. Therefore, we performed a combination study to
453 understand the effect of ZnNPs-PK on the vancomycin sensitivity to MRSA. We found ¼
454 MIC concentrations of both antibacterial in combination completely inhibit the growth of
455 MRSA as compared with individual treatment (Fig.8B). Therefore, this signifies that ZnNPs-
456 PK may intensify the sensitivity of vancomycin to MRSA.

457 **3.9 Cytotoxicity of ZnNPs-PK on HaCaT (Keratinocytes) cell line**

458 Cytotoxicity of ZnNPs-PK on HaCaT (Keratinocytes) cell line was determined by MTT
459 assay. The IC₉₀ (concentration at which 90% decrease in cell viability was observed) value
460 was found to be 400 µg/ml. The cytotoxic concentration of nanoparticles was found to be 5-
461 fold higher than that of MIC for bacteria (Fig.7C). Selectivity of ZnNPs-PK was calculated
462 by taking the ratio of IC_{90HaCaT}/MIC_{MRSA} and it was found to be 5.33 [37].

463 **Discussions:**

464 Organisms like MRSA are primarily known for its skin infections. It is a foremost reason of
465 bacteraemia and infective endocarditic problems additionally osteo-articular, skin and

466 flexible tissue damage[38]. As MRSA is considered as a well-known MDR strain, it is
467 tremendously difficult to be treated to normal ranges of antibiotics. Moreover, next
468 generations of antibiotics also impart toxic side effects on human body[39]. The treatment is
469 further complicated as MRSA are known as strong biofilms producer[40]. The biofilms
470 formations not only increase the resistant against the antibiotic but also prevent antagonistic
471 activities of immune cells of the host[41]. Under these circumstances, it has become very
472 important to produce an alternative way which has efficient antimicrobial as well as
473 antibiofilm effect on the respective strain though having low toxicity. In this study, initially
474 we have been able to produce zinc oxide nanoparticles (ZnNPs). ZnNPs are considered as
475 less toxic, biosafe among the other metal nanoparticles[10]. Further, these nanoparticles have
476 been doped with antibacterial enzyme pancreatin. The initial process of this study includes
477 the characterization of the formed nanoparticles which involves analytical tests like DLS,
478 UV-visible spectroscopy. Both DLS and UV spectra analysis assure the presence of doped
479 pancreatin on the surface of zinc-oxide nanoparticles. The lower MIC value of ZnNPs-PK
480 than ZnNPs suggest pancreatin doping increased the antimicrobial potential of nanoparticles.
481 Tolerance value indicated biocidal activity of ZnNPs-PK, which is further confirmed by
482 death kinetics assay. Inhibition of biofilm formation of pathogenic organisms is also an
483 important criterion for a comprehensive antimicrobial agent [42]. ZnNPs-PK was found to
484 impaired initial and mature biofilms formation of MRSA. It can be hypothesized that,
485 amylase lipase and protease activity of pancreatin degrade the carbohydrate and protein rich
486 biofilms produced by MRSA. After degradation of the biofilms it become easier for the
487 ZnNPs to invade and shows antagonistic activity on the sessile bacterial cell. Motility is an
488 important criterion of bacteria for its initial attachment with substratum and following biofilm
489 formation to disease progression[32]. Therefore, antimotility activity of antibacterial agent
490 should provide an additional advantage for disease management. In our study, ZnNPs-PK

491 inhibit both sliding motility and colony spreading activity of staphylococcus and may
492 consequently inhibit the biofilm formation as reported previously. Like motility, EPS
493 production also plays an important role for bacterial adhesion, adaptation and biofilm
494 formation [33]. EPS production significantly reduced in ZnNPs-PK treated MRSA, which
495 might be the reason for the reduction of biofilm of MRSA. MRSA produce variety of
496 virulence factors for the disease progression [43]. Therefore, inhibition of one or two
497 virulence factors is not enough for a comprehensive treatment. ZnNPs-PK was also found to
498 diminish staphyloxanthin secretion and eventually increase susceptibility of MRSA to
499 oxidative stress and host immune system. Not only that, other two virulence factors those are
500 important for survival within host, such as the haemolytic and plasma clumping activity were
501 significantly reduced in ZnNPs-PK treated MRSA give an idea of anti-infective nature of the
502 nanoparticles. This anti-infective nature of nanoparticles was further assessed by porcine skin
503 model. Where we found bioburden was significantly reduced in ZnNPs-PK treated MRSA
504 infected skin model as compared with control. All these data suggest ZnNPs-PK is an
505 effective antibacterial, anti-biofilms, anti-virulence and anti-infective agent against MRSA.
506 Furthermore, it is important to understand the mechanism associated with antibacterial
507 activities of ZnNPs-PK on MRSA. It has been previously reported that ZnNPs induced ROS
508 generation and targets bacterial membrane [44]. In our study we also found ZnNPs-PK
509 treatment induced significant reduction of membrane potential indicating it may target
510 bacterial cell membrane. Bacterial cell membrane plays a vital role for the survival and
511 growth of the bacteria [45]. Reduction of membrane potential also increased the ROS
512 generation in treated cells and that is similar to previous reports [46]. ROS may oxidize
513 different cellular constituent including proteins, nucleic acid and lipid of the cell membrane.
514 Moreover, lipid oxidation may increase permeability of membrane as we found significant
515 uptake of PI in treated cells than untreated cells. Ultimately damage to membrane may leads

516 to release of cellular constituents and death of the bacterial cells. This clinical MRSA was
517 found to be intermediate resistance to vancomycin. We found combination treatment of $\frac{1}{4}$
518 MIC of both vancomycin and ZnNPs-PK completely inhibited the growth of bacteria. This
519 may indicate synergistic activity of ZnNPs-PK and vancomycin in combination against
520 MRSA. Finally, it is important to understand biosafe nature of the prepared nanoparticles. the
521 IC₉₀ concentration of nanoparticles were five times higher than the MIC of MRSA. The ratio
522 of IC₉₀_{HaCaT} and MIC_{MRSA} showed that ZnNPs-PK is selectively cytotoxic towards MRSA as
523 the concentration required to inhibit bacterial growth is much lesser, i.e., 5-fold less than
524 IC₉₀_{HaCaT}.

525 **Conclusions:**

526 This study concluded that ZnNPs-PK possesses bactericidal, anti-biofilms, anti-motility and
527 anti-virulence properties against MRSA. ZnNPs-PK targets cell membrane and increased
528 oxidative stress as its mode of action against MRSA. It increased the vancomycin sensitivity
529 of our clinical MRSA strain. ZnNPs-PK was found to nontoxic with selective bactericidal
530 properties. In a nutshell, ZnNPs-PK can be used for comprehensive treatment of diseases
531 associated with MRSA.

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537 **Conflict of interest:**

538 The authors declare that there is no conflict of interests regarding the publication of this
539 paper.

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- 677

678 5. Figure Legends:

679 **Fig.1** – Characterizations of ZnNPs and ZnNPs-PK. (A) Comparative UV-VIS spectra of
680 pancreatin, ZnNPs and ZnNPs-PK by UV-spectrophotometer. (B) Comparative analysis of
681 ZnNPs (B1) and ZnNPs-PK(B2) by Dynamic Light Scattering (DLS).

682 **Fig.2**– Antimicrobial activities of ZnNPs-PK on *MRSA*. (A) Graphical representation of
683 comparative antimicrobial activity of ZnNPs and ZnNPs-PK (B) Death Kinetics of *MRSA* in
684 presence and absence of ZnNPs-PK.

685 **Fig.3** – Anti-biofilms activity by ZnNPs-PK against *MRSA*. (A) Tabular representation of
686 effect of ZnNPs-PK on the viability of sessile cells and biofilm biomass of initial and mature
687 biofilms (B) Light (B1 and B2) and confocal microscopic (B3 and B4) observation of ZnNPs-
688 PK induced biofilm inhibition

689 **Fig.4** – Anti-motility activity by ZnNPs-PK against *MRSA* (A) Pictorial representation of
690 inhibition of sliding motility (A1 and A2) and colony motility (A3 and A4) of *MRSA* by
691 ZnNPs-PK.

692 **Fig.5** – Anti-virulence properties of ZnNPs-PK against *MRSA* (A) Pictorial representation of
693 inhibition of slime production of *MRSA* by ZnNPs-PK. (A1) represents untreated cells. (A2)
694 represents treated cells. (B) Pictorial representation of plasma clamping ability (A3 and A4)
695 of *MRSA* by ZnNPs-PK. (B1) blood plasma with untreated *MRSA* cells. (B2) blood plasma
696 with treated *MRSA* cells.

697 **Fig.6** - (A) Tabular representation of effect of ZnNPs-PK on exopolysaccharide production,
698 staphyloxanthin production and hemolytic activity of *MRSA*. (B) ROS generation was flow
699 cytometrically evaluated in untreated and ZnNPs-PK treated *MRSA* cells by using DCFDA.

700 **Fig.7** – Mechanism of action of ZnNPs-PK against *MRSA* (A) Membrane potential was flow
701 cytometrically evaluated in untreated and ZnNPs-PK treated *MRSA* cells by using Rh123 (B)

702 Cell membrane damage was flow cytometrically evaluated in untreated and ZnNPs-PK
703 treated MRSA cells by PI uptake

704 **Fig.8** – Ex vivo study and combination study of ZnNPs-PK (A) Graphical representation of
705 bioburden of MRSA cells untreated and treated with ZnNPs-PK in porcine skin model.
706 (B)Graphical representation of combination activity of ZnNPs-PK and Vancomycin against
707 MRSA

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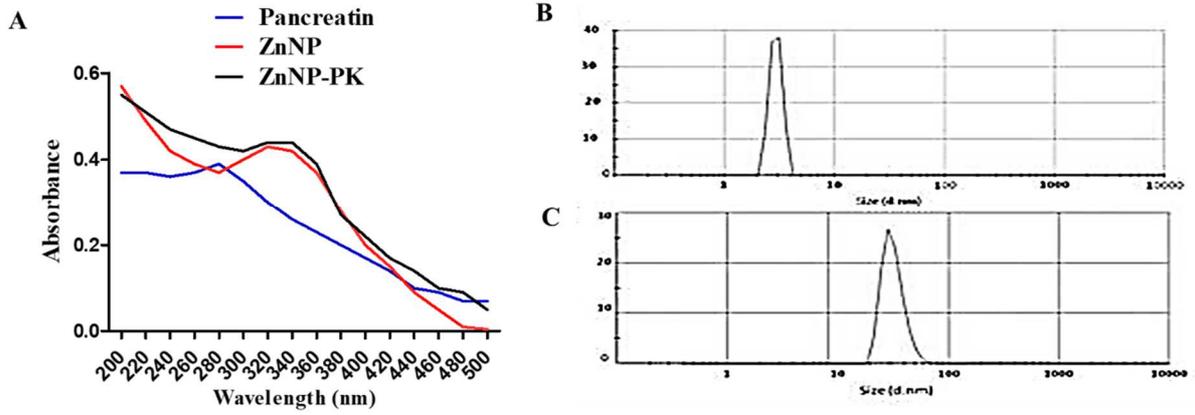
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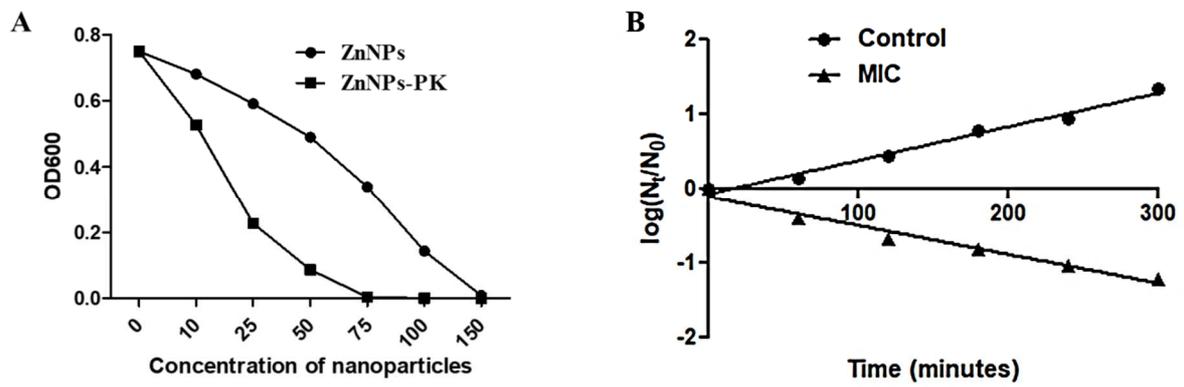
725 **Figures:**



726

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Fig.1



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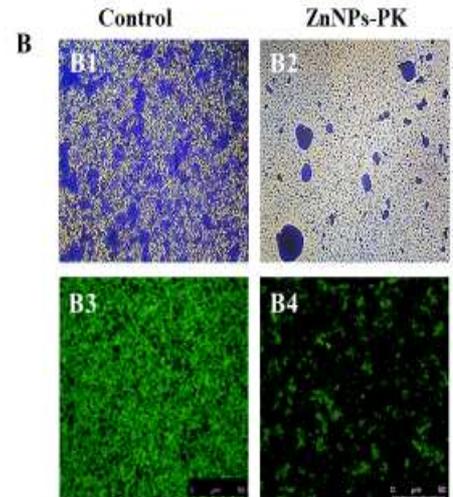
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Fig.2

A

	Initial Biofilm		Mature Biofilm	
	CFU/ml	Biomass	CFU/ml	Biomass
Control	8.38 ± 0.67	100	8.61 ± 0.23	100
½ MIC	6.6 ± 0.21	38 ± 3.2	6.8 ± 0.07	51 ± 1.1
MIC	4.2 ± 0.31	17 ± 2.7	5.1 ± 0.15	32 ± 2.4

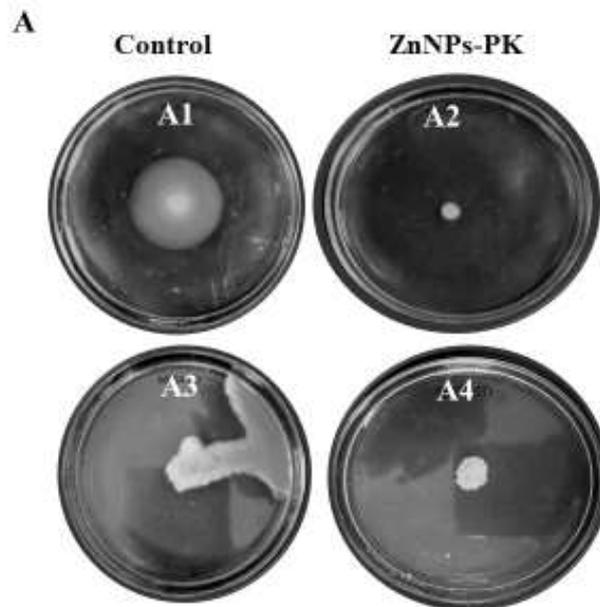


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Fig.3

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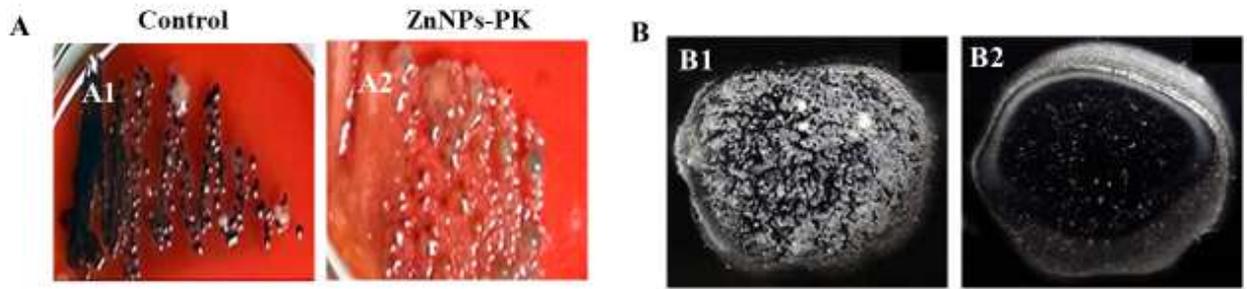


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Fig. 4

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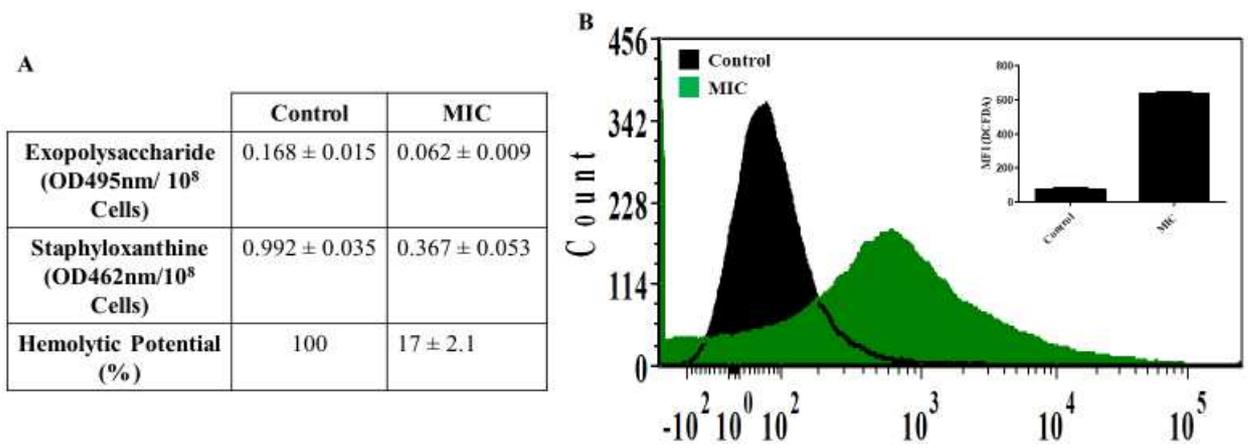


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Fig. 5

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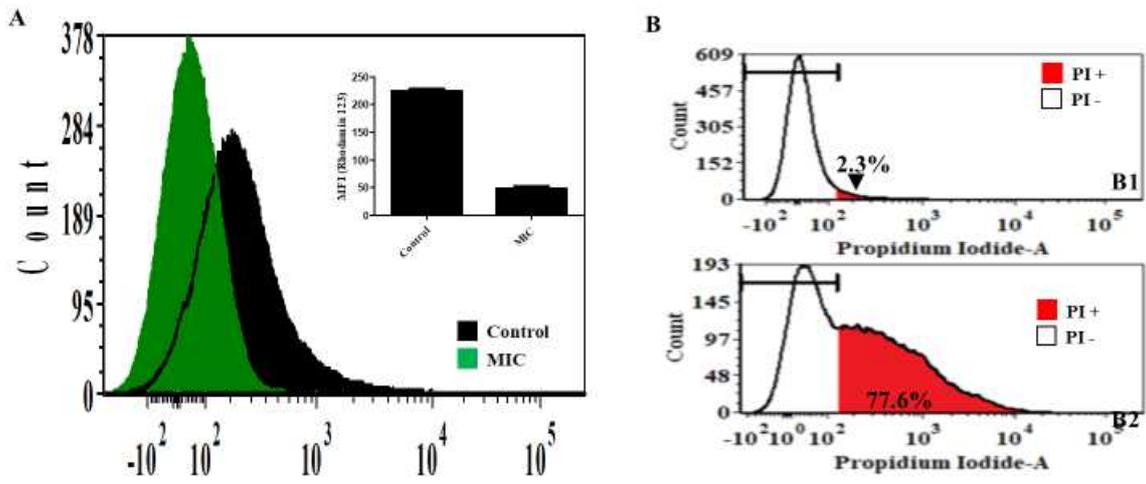
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Fig.6

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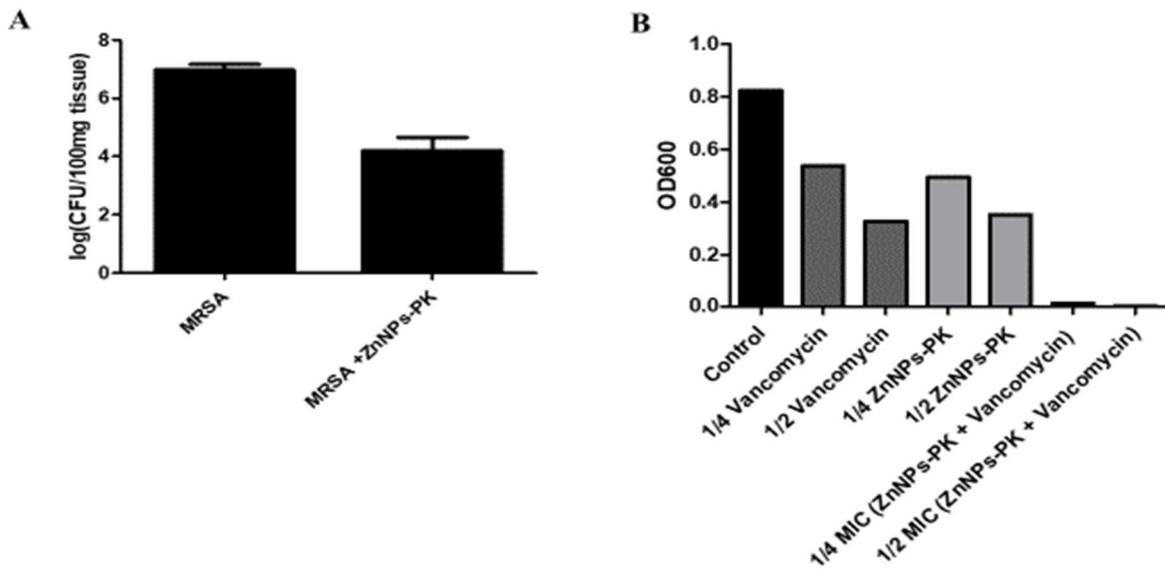
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745

746

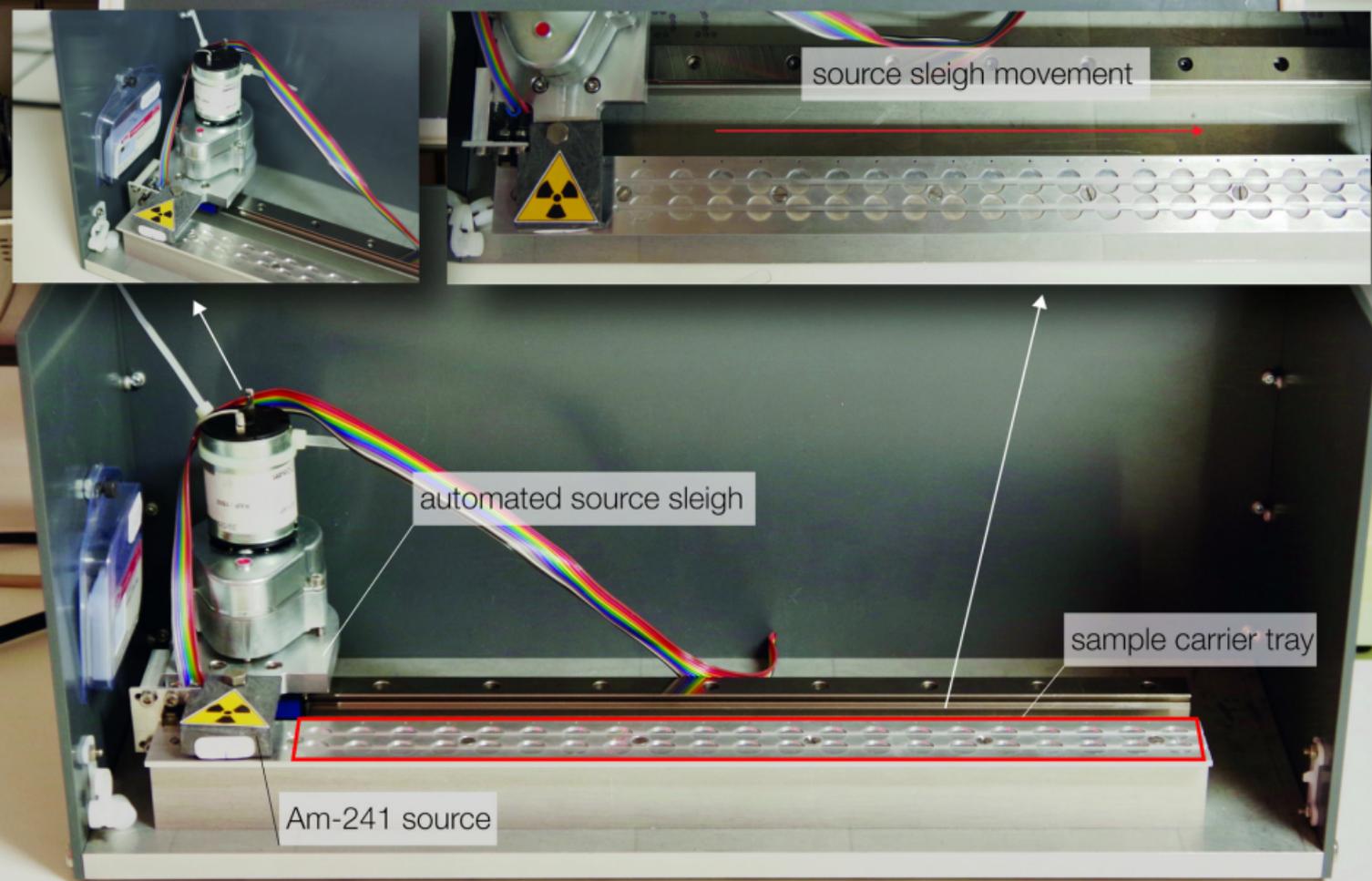
Fig.7



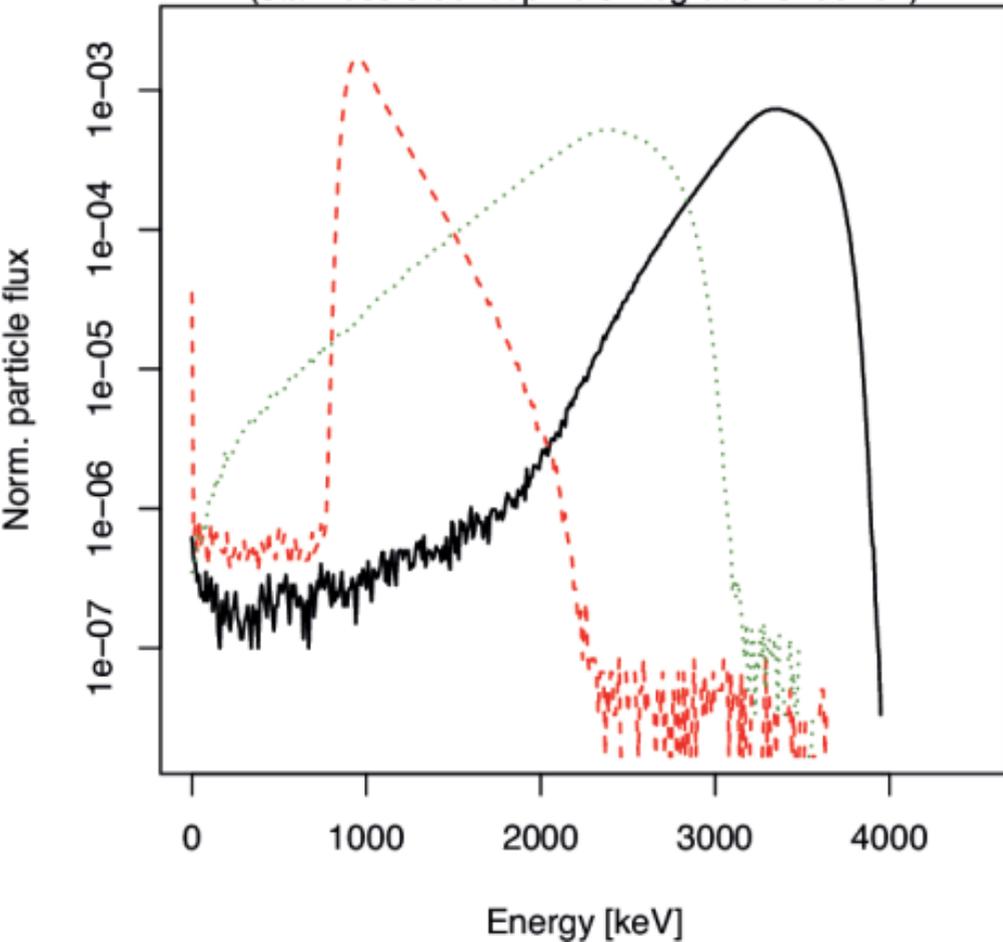
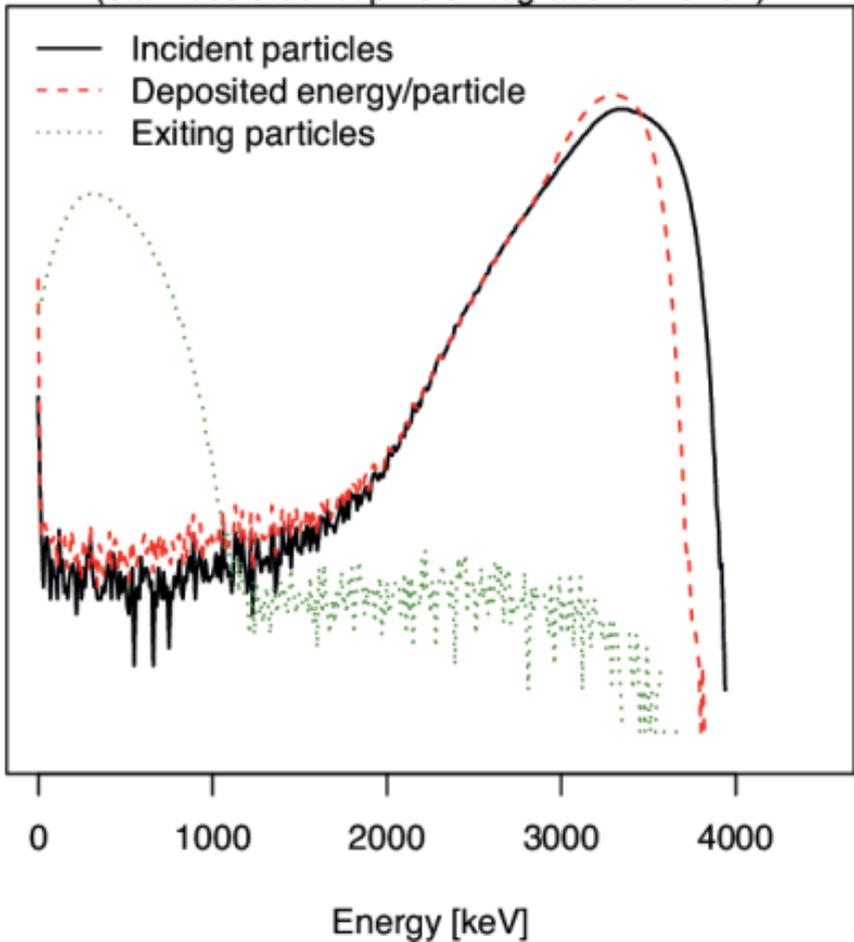
747

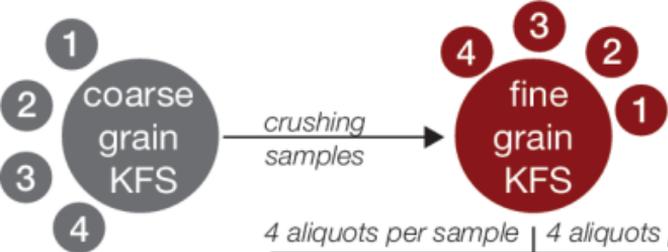
748

Fig. 8



Dimension: 115 x 270 x 124 mm (length x width x height)

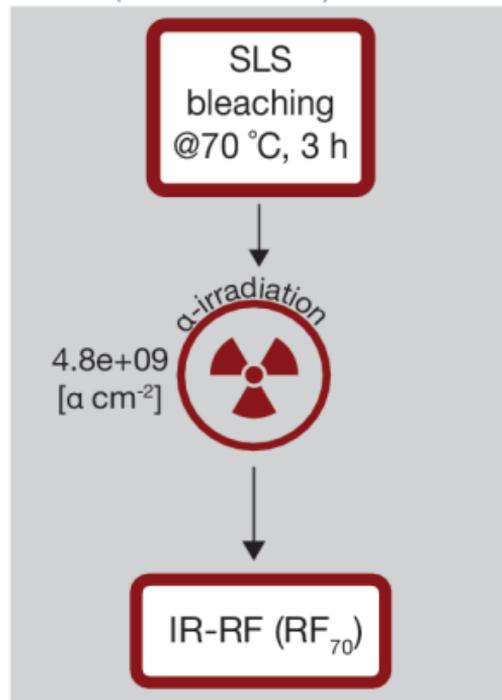
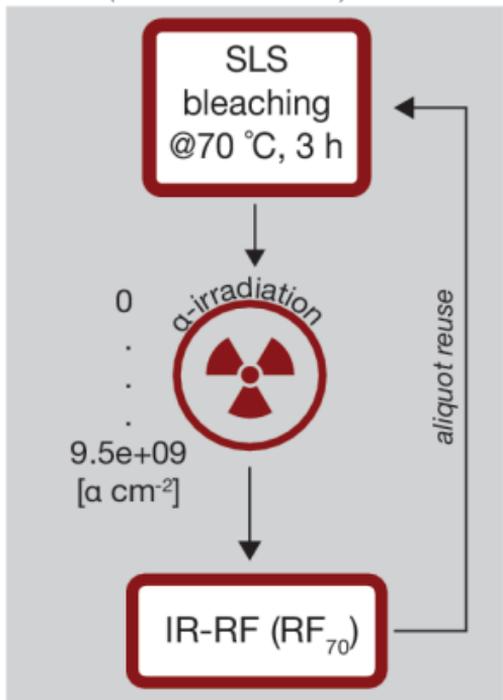
A**4 μm** (stainless steel cup I disintegrations: $6\text{e}+07$)**B****11 μm** (stainless steel cup I disintegrations: $4\text{e}+07$)



4 aliquots per sample | 4 aliquots per sample

Series 1 (before 2017-11-05)

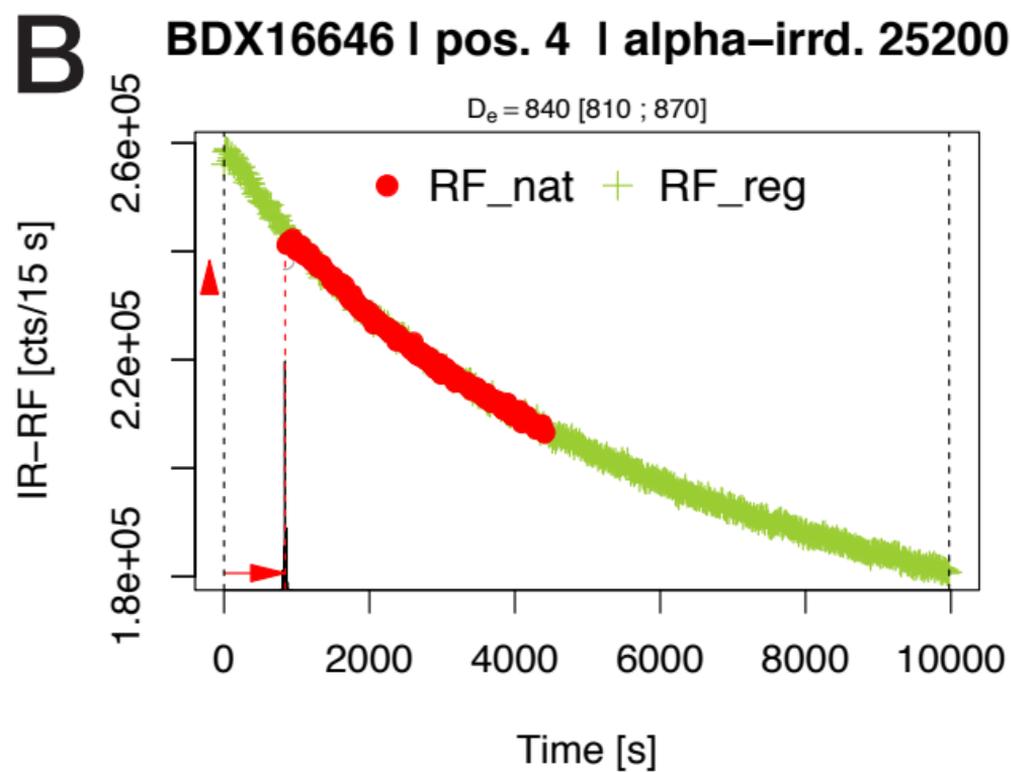
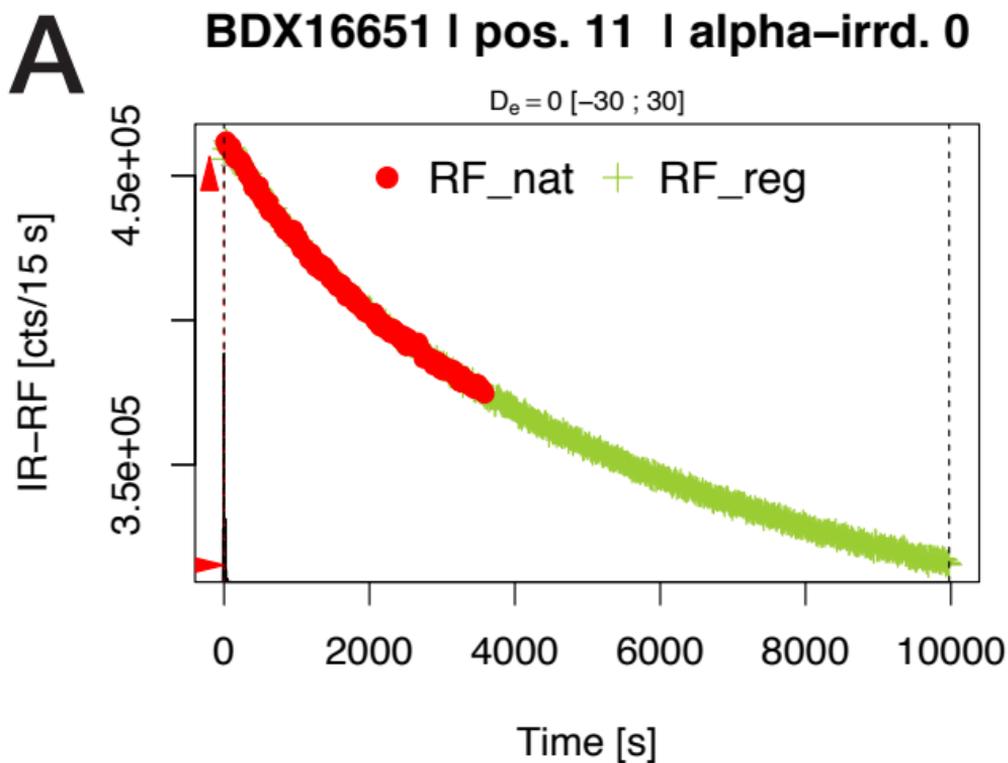
Series 2 (after 2017-11-05)



n = 68

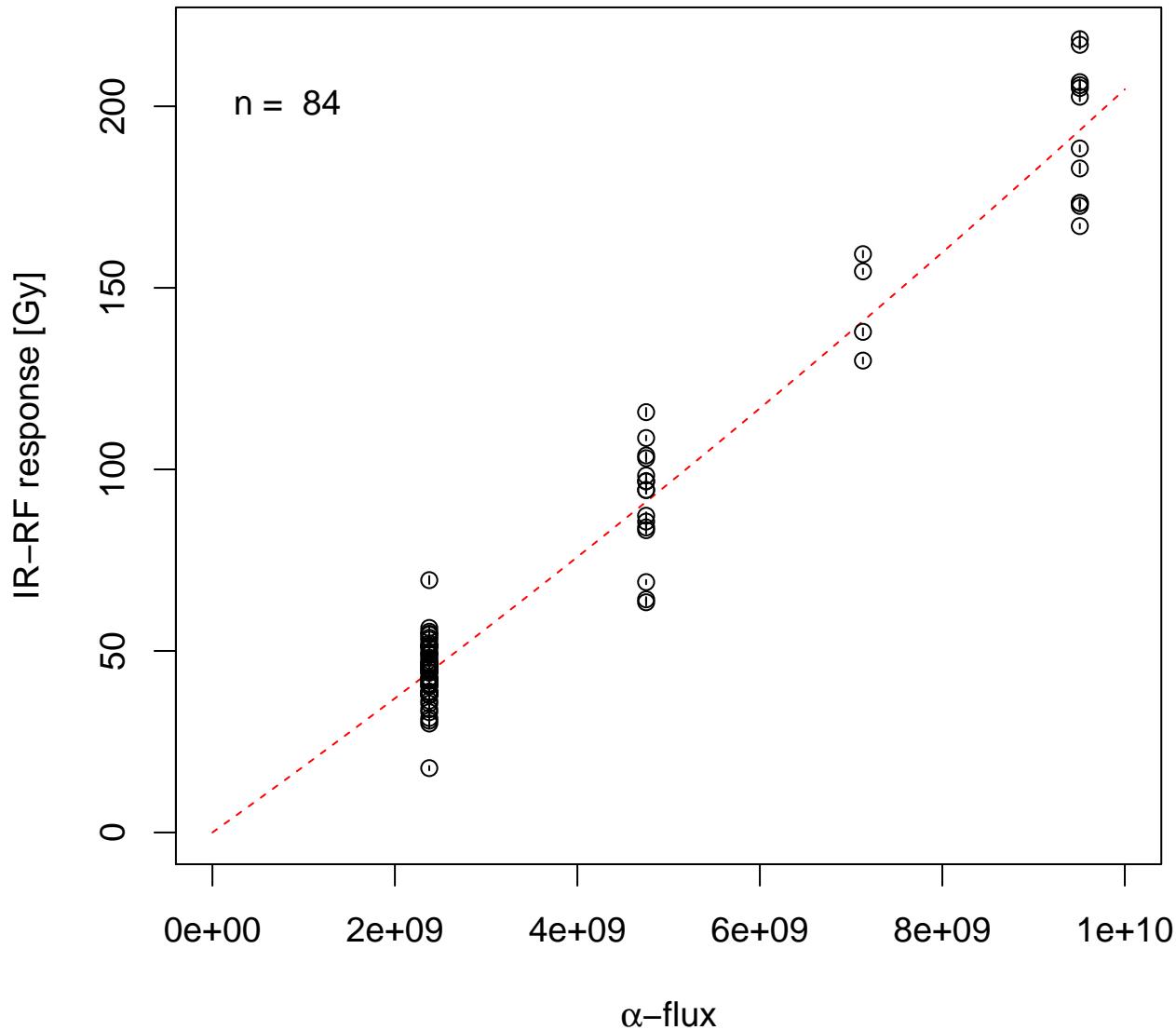
Combined summary

n = 16

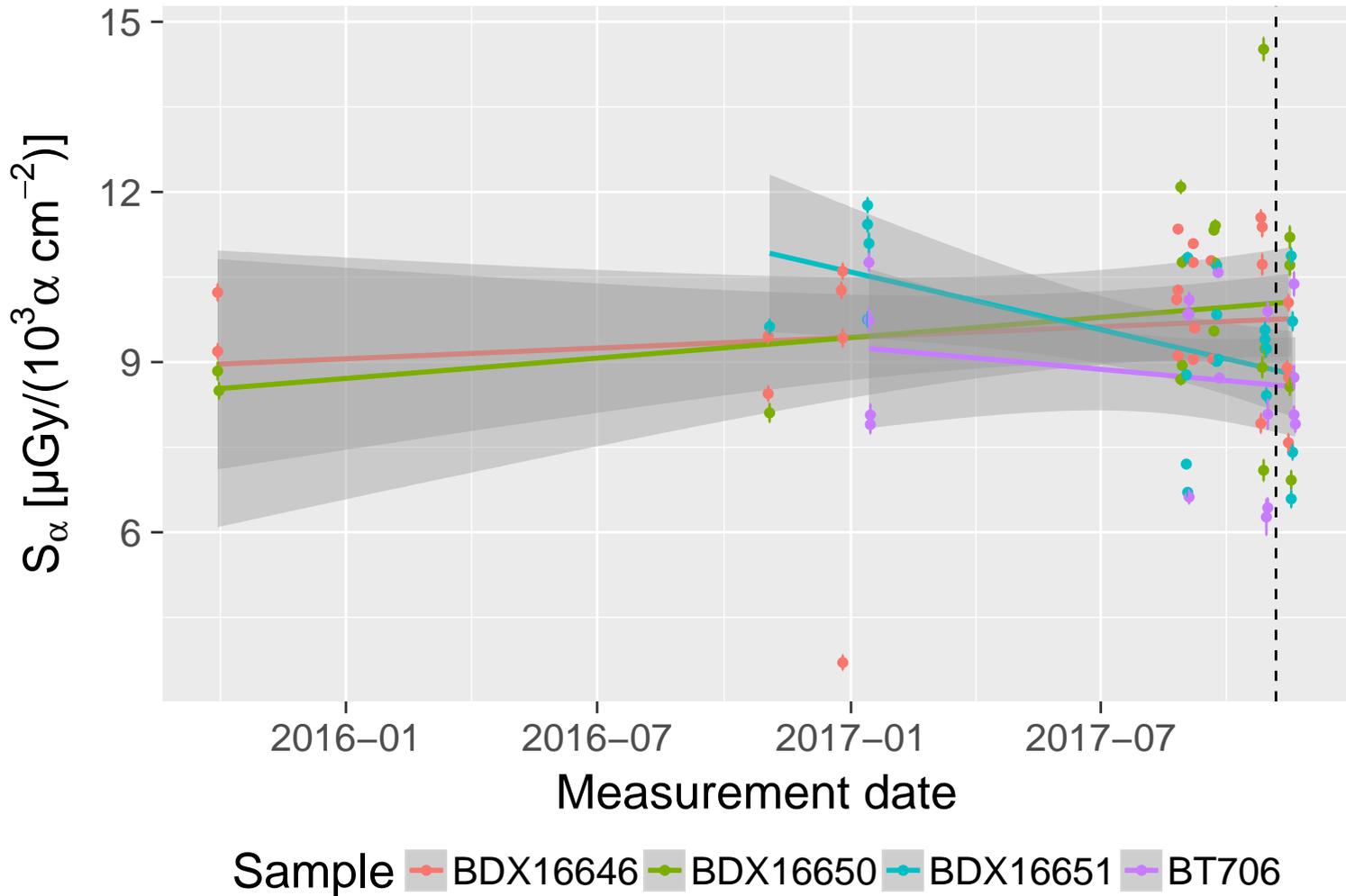


Alpha-flux Response Curve

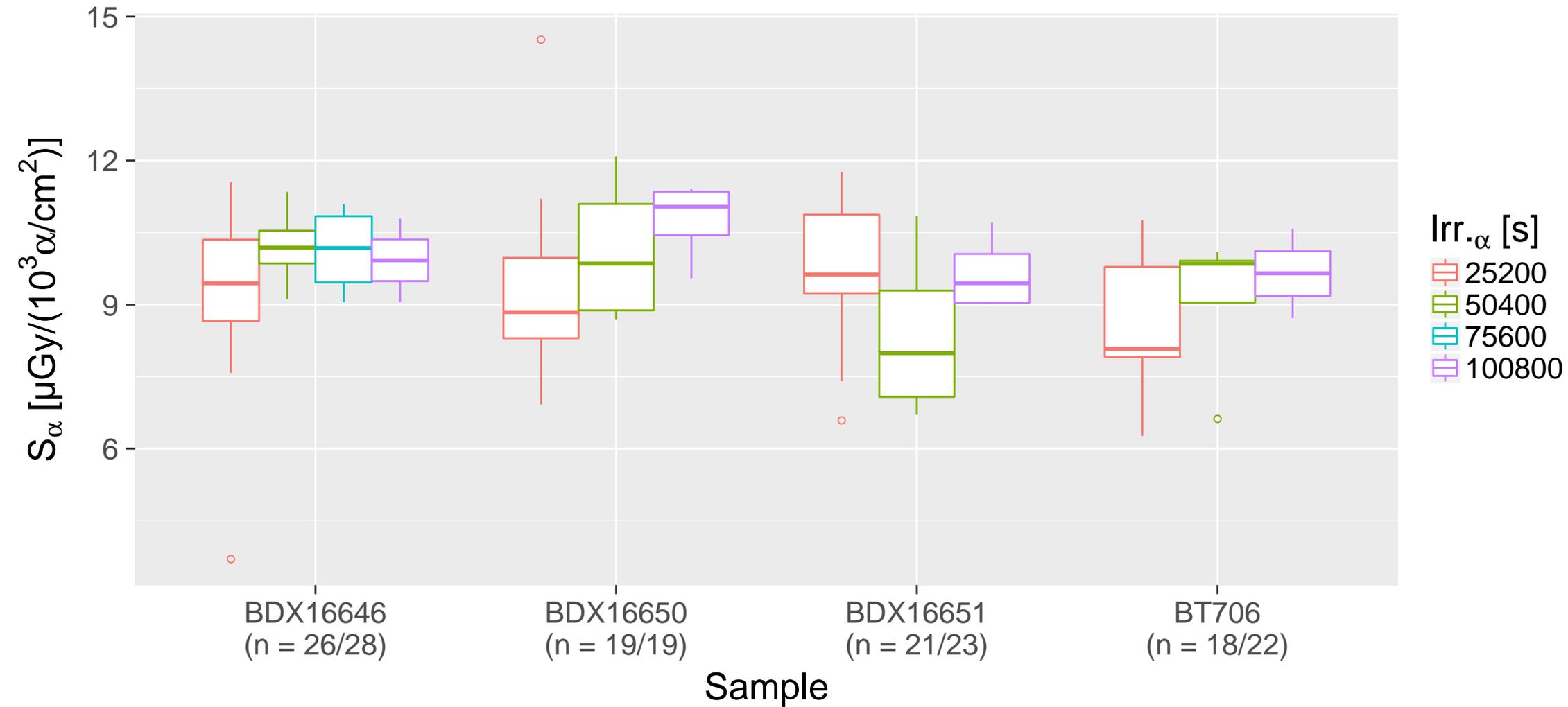
$$\text{fit : } 1.797618\text{e-}08 * x + 2.490935\text{e-}19 * x^2$$



S_α -values over Time

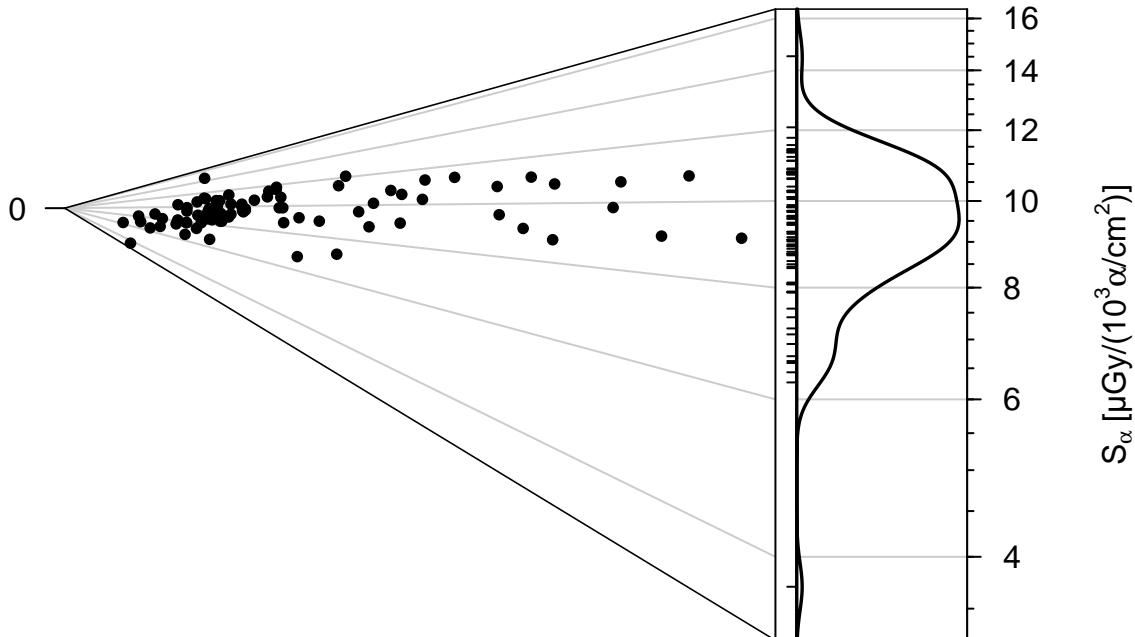


Summary S_α values



n = 84 | mean = 9.26 | abs. sd = 1.62 | rel. sd = 17.23 %

Standardised estimate



Relative standard error (%)

2

1

0.7

0.5

0.4

0

50

100

150

200

250

0

0.241

Precision

Density (bw 0.062)

Table 1

SAMPLE	N	S_{α} [$\mu\text{Gy}/(10^3\alpha \text{ cm}^{-2})$]	$SE(S_{\alpha})$ [$\mu\text{Gy}/(10^3\alpha \text{ cm}^{-2})$]	CV [%]
BDX16646	26/28	10.0	1.2	12.4
BDX16650	19/19	10.0	1.3	13.0
BDX16651	21/23	9.2	1.2	12.8
BT706	18/22	9.0	1.2	13.2

Note: All values are given as error weighted mean. SE is the standard deviation of the sampling distribution.