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Title: Mixed Protein-DNA Gel Particles for DNA Delivery: Role of Protein Composition and Preparation Method on Biocompatibility

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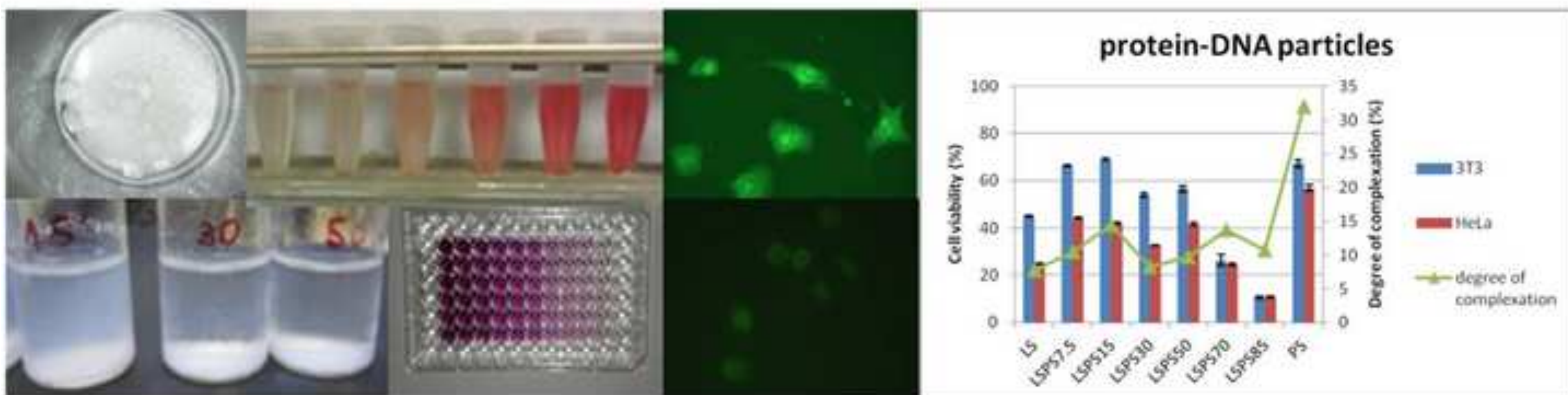
*Graphical Abstract (for review)



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Mixed Protein-DNA Gel Particles for DNA Delivery: Role of Protein Composition and Preparation Method on Biocompatibility

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11

12 ## Abstract

13 Mixtures of two cationic proteins were used to prepare protein-DNA gel particles, employing
14 associative phase separation and interfacial diffusion (Morán et al., 2009a). By mixing the two
15 proteins, we have obtained particles that displayed higher loading efficiency and loading capacity
16 values than those obtained in single-protein systems. However, nothing is known about the
17 adverse effects on haemocompatibility and cytotoxicity of these protein-DNA gel particles. Here,
18 we examined the interaction of protein-DNA gel particles obtained by two different preparation
19 methods, and their components, with red blood cells and established cells. From a haemolytic
20 point of view, these protein-DNA gel particles were demonstrated to be promising long-term blood-
21 contacting medical devices. Safety evaluation with the established cell lines revealed that, in
22 comparison with proteins in solution, the cytotoxicity was reduced when administered in the
23 protein-DNA systems. In comparison with large-sized particles, the cytotoxic responses of small-
24 sized protein-DNA gel particles showed to be strongly dependent of both the protein composition
25 and the cell line being the tumour cell line HeLa more sensitive to the deleterious effects of the
26 mixed protein-based particles. The observed trends in haemolysis and cell viabilities were in
27 agreement with the degree of complexation values obtained for the protein-DNA gel particles
28 prepared by both preparation methods.

29 **Keywords:** DNA gels, particles, size, haemolysis, *in vitro* cytotoxicity
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34 1. Introduction

35

36 A general understanding of the interactions between DNA and oppositely charged agents, and in
37 particular of phase behaviour, has provided a basis for developing novel, DNA-based materials,
38 including gels, membranes and gel particles (Costa et al., 2008; Lindman et al. 2009, Lindman et
39 al., 2010) We prepared novel DNA gel particles employing associative phase separation and
40 interfacial diffusion. By mixing solutions of DNA (either single- (ssDNA) or double-stranded
41 (dsDNA)) with solutions of different cationic agents, such as surfactants, proteins and
42 polysaccharides, the possibility of forming DNA gel particles without adding any kind of cross-linker
43 or organic solvent has been confirmed (Morán et al, 2010, Morán et al., 2013 and references
44 therein).

45 A novel nonviral vector for gene therapy is recognised as successful if it is biocompatible, capable
46 of interacting with DNA, able to form sufficiently small particles which can be formulated
47 reproducibly, endocytosed, able to protect the complexed DNA from degradation during transport,
48 and capable of delivering DNA to the target tissue in sufficient quantity (Ledley, 1996; Pack et al.,
49 2000; Davis, 2002).

50 Among the different cationic agents studied, the cationic surfactants offered particularly efficient
51 control of the properties of these DNA-based particles (Morán et al., 2007a; Morán et al., 2007b)
52 However, the cytotoxicity of quaternary ammonium surfactants is well known (Lasic, 1997).
53 Proteins as the matrices for drug delivery particles have many advantages including
54 biodegradability, biocompatibility, and amenability to surface modification (Kratz, 2008). Drug
55 carriers using albumin as matrices for the delivery of small molecule drugs and biological cargos,
56 such as plasmid DNA and siRNA, are being previously studied (Rhaese et al., 2003; Hawkins et
57 al., 2008; Abbasi et al., 2011). However, in all cases, the presence of cationic polyelectrolytes is

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58 needed to condense the nucleic acids to efficiently mediate gene transfer (Rhaese et al., 2003;
59 Abbasi et al., 2011).

60 DNA gel particles have been prepared using the cationic functionality of a cationic protein.
61 Lysozyme has been used as carrier to form DNA gel particles by interfacial diffusion (Morán et al.,
62 2007a) The obtained particles, however, were not able to protect the secondary structure of the
63 condensed DNA and an important burst release stage was observed when the kinetics of DNA
64 release from these protein-DNA gel particles were determined. In a recent study, two cationic
65 proteins, lysozyme and protamine sulphate, were used as biocompatible carriers to form DNA gel
66 particles by interfacial diffusion (Morán et al., 2009a). The particles were characterised with respect
67 to the degree of DNA entrapment, swelling and dissolution behaviour, surface morphology,
68 secondary structure of DNA in the particles and kinetics of DNA release, and the study
69 demonstrated that DNA was effectively entrapped in the mixed protein solutions, protecting its
70 secondary structure. A significant increase in the degree of effective entrapment of DNA was
71 achieved by mixing the two proteins. The magnitude of DNA release was controlled and controlled
72 release systems were achieved by changing the lysozyme/protamine ratio in the protein solution
73 where particles were formed.

74 At a consensus conference of the European Society for Biomaterials in 1986, the word
75 “biocompatibility” was defined as “the ability of a material to perform with an appropriate host
76 response in a specific application”. With the rapid development of biomaterials, the scope of
77 “biocompatibility” has been widely broadened. On the basis of designing DNA gel particles for
78 therapeutic purposes, we use biocompatibility to include the deleterious effects caused by the DNA
79 gel particles, covering the *in vitro* haemolytic and cytotoxic assessments. Currently, nothing is
80 known about the adverse effects on biocompatibility of these protein-DNA gel particles. One
81 drawback of these DNA gel particles, in toxicological terms, is the need for a cationic compound,
82 which may cause some cellular damage. A recent study (Morán et al., 2012) indicate, however,
83 that the effect of the cationic surfactants can be modulated when administered in the DNA gel
84 particles, unlike what happens in aqueous solution. This modulation is due to the strong interaction

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85 between the surfactant and the biopolymer, which leads to a very slow release of the surfactant
86 from the vehicle.

87 Safety evaluation of new products or ingredients destined for human use is crucial prior to
88 exposure. Therefore, rapid, sensitive and reliable bioassays are required in order to examine the
89 toxicity of these substances. Established cell lines are useful alternative test systems for
90 toxicological studies of this kind (Crespi, 1995); however, they must be chosen with care with
91 regard to their origin (Jondeau, et al., 2006). Moreover, cytotoxicity assays are among the most
92 common *in vitro* endpoints used to predict the potential toxicity of a substance in a cell culture
93 (Martínez et al., 2006). The size of particles plays an important role regarding *in vitro* and *in vivo*
94 applications. Even particles of the same material can show completely different behaviour due to,
95 for example, slight differences in surface coating, charge or size. The particle size determines the
96 efficiency of cellular uptake and subsequent intracellular processing (Rejman et al., 2004).

97 Nano-sized materials have a high potential in technical and medical applications provided they are
98 not toxic. Despite the significant scientific interest and promising potential, the safety of
99 nanoparticulate systems remains a growing concern, considering that biological applications of
100 nanoparticles could lead to unpredictable effects. The prediction of toxicity is difficult, but
101 cytotoxicity screening, which is routinely used in drug screening, gives a good indication of
102 potential adverse effects in cells. As a general rule, nano-sized materials show higher reactivity
103 than bulk materials of the same composition. Size, surface charge, and hydrophobicity interact in
104 complex ways and have a pronounced influence on biocompatibility. Aggregation in physiological
105 fluids is often observed. Therefore, toxicity data must be interpreted in the context of the
106 physicochemical characteristics of the nano-sized materials (Fröhlich et al., 2012).

107 Currently, there are no specific testing requirements for nanotechnology products, and therefore,
108 researchers took liberal approaches to studying toxicity (Boverhof and David, 2010; Robbens et al.,
109 2010). Moreover, it is worth noting that, because of the expense of animal testing in toxicology and
110 pressure from both the general public and government to develop alternatives to *in vivo* testing, i*n*

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111 *vitro* cell-based models may be more attractive for preliminary testing of nanomaterials (Hillegass
112 et al. 2009).

113 In this context, the purpose of the present study was to prepare and characterise the
114 haemocompatibility of and cytotoxicity response to protein-DNA gel particles formed by mixing
115 double-stranded DNA (dsDNA) with lysozyme/protamine sulphate mixtures using two different
116 approaches. The interaction of these protein-DNA gel particles and their components with
117 erythrocytes, non-tumour (3T3 fibroblast) and tumour (HeLa) cell lines is described, using the
118 imposed variations in protein composition and the size of the final particles, as a consequence of
119 the different preparation method, as controlling parameters.

120 2. Experimental section

121 2.1. Materials

122 The sodium salt of deoxyribonucleic acid (DNA) from salmon testes with an average degree of
123 polymerisation of ≈2000 base pairs (bp) was purchased from Sigma and used as received. The
124 DNA concentrations were determined spectrophotometrically, assuming that for an absorbance of
125 1 at 260 nm, a solution of dsDNA has a concentration of 50 μg mL^{-1} (Sambrook et al., 1889). All
126 DNA concentrations are given in molarity per phosphate group, i.e., molarity per negative charge.
127 The absorbance ratios at 260 and 280 nm of the stock solutions were found to be between 1.8 and
128 1.9, which suggested the absence of proteins (Saenger, 1984). Lysozyme from chicken egg white
129 (LS), with a molecular mass of 14.3 kDa, protamine from salmon in the sulphate salt (PS), with a
130 molecular mass of 5.1 kDa, and Tris base were purchased from Sigma and used as received.
131 N,N,N',N'-tetramethylacridine-3,6-diamine (acridine orange (AO)) was supplied by Molecular
132 Probes (Invitrogen).

133

134 2.2. Particle Preparation

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135 The dsDNA stock solutions were prepared in 10 mM NaBr to stabilise the DNA secondary structure
136 in its native B-form conformation. LS, PS, or mixtures of both were dissolved in a PBS buffer (pH
137 7.4). Table 1 summarises the composition of the protein systems studied.

138 **[Table 1 here]**

139 ### *2*.2.1. Protein-DNA gel particles prepared by the dropwise addition method

140 Particles were prepared at a DNA and protein system ratio equal to 1, R= [DNA]/[P+], where [P+] is
141 the concentration of the corresponding protein system (concentrations determined per charge). In
142 all cases, [DNA] was equal to 60 mM. DNA solutions were added dropwise via a 22-gauge needle
143 into gently agitated protein solutions (2 mL). Under optimal conditions, droplets from DNA solutions
144 instantaneously gelled into discrete particles upon contact with the protein solution. Thereafter, the
145 particles were equilibrated in the solutions for a period of 2 h at room temperature. After this
146 period, the particles formed were separated by filtration through a G2 filter and washed with 5 x 8
147 mL PBS to remove excess salt.

148 ### 2.2.2. Protein-DNA gel particles prepared by the nebulisation method

149 Particles were prepared at a DNA and protein system ratio equal to 1. In this case, [DNA] was
150 equal to 2.5 mM. Higher concentrations of DNA produced high-viscosity solutions, rendering them
151 inconvenient systems for the nebulisation process. The Comp Air NE-28-E nebuliser (Omrom)
152 employed enabled the generation of very fine droplets/aerosols with a MMAD (Mass Median
153 Aerodynamic Diameter) of 2.8 μm for NaF solution, independently measured at SolAero Ltd,
154 Canada, by Dr John Dennis, according to EN 13544–1. DNA solutions were nebulised and added
155 to gently agitated protein solutions (5 mL).

156

157 ## 2.3. Interaction with Erythrocytes

158 ### 2.3.1. Preparation of red blood cell suspensions

159 Rat blood was obtained from anaesthetised animals by cardiac puncture and drawn into tubes
160 containing EDTA. This procedure was approved by the Ethical Committee for Animal Research of

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161 the University of Barcelona. Serum was removed from the blood by centrifugation at 3,000 rpm
162 (Megafuge 2.0 R Heraeus Instruments) at 4 º C for 10 min, and subsequent suction. The red blood
163 cells were then washed three times at 4 º C by centrifugation at 3,000 rpm with isotonic saline PBS
164 solution, containing 22.2 mmol L^{-1} Na_2HPO_4, 5.6 mmol L^{-1} KH_2PO_4, 123.3 mmol L^{-1} NaCl in distilled
165 water (pH 7.4). Following the last wash, the cells were diluted to ½ of their volume with isotonic
166 phosphate buffer solution (PBS) (cell density of 8 x 10^9 cell mL^{-1}).

167 **2.3.2. Haemolysis assay**

168 The membrane-lytic activity of the systems was examined by haemolysis assay. Firstly, haemolytic
169 response to the different proteins in solution was tested. Thus, a series of different volumes of
170 protein solution (10 mg mL^{-1} in PBS), ranging from 10 to 200 µL, were placed in polystyrene tubes
171 and an aliquot of 25 µL of the erythrocyte suspension was added to each tube. The final volume
172 was 1 mL. The tubes were incubated at room temperature for 10 min under shaking conditions.
173 Following incubation, the tubes were centrifuged (5 min at 10, 000 rpm). The degree of haemolysis
174 was determined by comparing absorbance (540 nm) (Shimadzu UV-160A) of the supernatant with
175 that of the control samples totally haemolysed with distilled water. Positive and negative controls
176 were obtained by adding an aliquot of 25 µL of erythrocyte suspension to distilled water and
177 isotonic PBS solution, respectively.

178 In the case of the protein-DNA systems, two kinds of experiments were carried out. Either the
179 individual protein-DNA gel particles (dropwise addition method) or the dispersions containing the
180 protein-DNA gel particles (nebulisation method) or the protein solutions that were used to prepare
181 the corresponding protein-DNA gel particles were studied. In the case of protein-DNA particles
182 obtained by the dropwise addition method, individual DNA gel particles were placed in the tubes. In
183 the case of dispersions containing the protein-DNA gel particles obtained by the nebulisation
184 method (100, 200 or 300 µL of the corresponding solution) or the protein solutions prior to the
185 preparation of the corresponding protein-DNA gel particles (25 µL of the corresponding solution)
186 were added to each tube. In all cases, an aliquot of 25 µL of erythrocyte suspension was added to

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187 each tube. The final volume was 1 mL. The tubes were incubated at room temperature for different
188 times (10-360 min) under shaking conditions. At the same defined times, the incubated samples
189 were centrifuged (5 min at 10, 000 rpm). The degree of haemolysis was determined following the
190 same procedure as described above.
191 The correlation between the erythrocyte population and the haemolytic response in the presence of
192 the particles was established by counting the number of erythrocytes on each sample using a
193 Bürker-Türk counting chamber, covered by a cover slip and analyzed by a contrast microscope
194 (Olympus BX41). Simultaneously with the counting of the erythrocytes on each sample, studies of
195 erythrocyte agglutination were carried out.

196 **2.3.3. DNA release**

197 Simultaneously with the haemolysis assay experiments, DNA release from the protein-DNA gel
198 particles was determined. To this end, individual DNA gel particles were placed in the tubes and
199 isotonic PBS solution was added to a final volume of 1 mL. The tubes were incubated at room
200 temperature for the same defined times as in the case of the haemolysis assay experiments, under
201 shaking conditions. The concentration of dsDNA released into the supernatants was determined by
202 using the NanoPhotometer™ (Implen.)

203 **2.3.4. Protein release**

204 Furthermore, protein release from the protein-DNA gel particles was determined. To this end,
205 individual DNA gel particles were placed in the tubes and isotonic PBS solution was added to a
206 final volume of 1 mL. The tubes were incubated at room temperature for the same defined times as
207 in the case of the haemolysis assay experiments, under shaking conditions. Following incubation,
208 the concentration of solubilised protein in the supernatants was determined using the Bio-Rad
209 Protein Assay, based on the method described by Bradford (Bradford, 1986). This involves the
210 addition of an acidic dye to the protein solution and subsequent measurement at 595 nm with a
211 spectrophotometer. Comparison with a standard curve provides a relative measurement of protein
212 concentration.

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213 ## 2.4. Cell culture

214 The murine Swiss albino 3T3 fibroblast cell line and the human epithelial carcinoma HeLa cell line
215 were grown in DMEM medium (4.5 g L^{-1} glucose) supplemented with 10% (v/v) FBS, 2 mM L-
216 glutamine, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin at 37ºC, 5% CO_2. The 3T3 and
217 HeLa cells were routinely cultured in 75 cm^2 culture flasks and were trypsinised using trypsin-
218 EDTA when the cells reached approximately 80% confluence.

219 ### 2.4.1.Cytotoxicity assays

220 The cytotoxic effect of the systems was measured by tetrazolium salt MTT assay (Mosmann,
221 1983). 3T3 and HeLa cells were seeded into the central 60 wells of a 96-well plate at a density of 1
222 x 10^5 and 5 x 10^4 cells $mL^{-1,}$ respectively. After incubation for 24 h under 5% CO_2 at 37ºC, the
223 spent medium was replaced in the wells with 100 µL of fresh medium supplemented with 5% FBS
224 containing protein solution at the required concentration range (50-2000 µg mL^{-1}).

225 In the case of the protein-DNA systems, two kinds of studies were carried out. Either the individual
226 protein-DNA gel particles (dropwise addition method) or the dispersions containing the protein-
227 DNA gel particles (nebulisation method) or the protein solutions that were used to prepare the
228 corresponding protein-DNA gel particles were studied. In the case of the individual protein-DNA gel
229 particles, 100 µL of fresh medium supplemented with 5% FBS was added in each well, and then
230 individual DNA gel particles were placed in them. In the case of the dispersion containing the
231 protein-DNA gel particles or the protein solutions prior to the preparation of the corresponding
232 protein-DNA gel particles, 100 µL of each system diluted 1:1 in fresh medium supplemented with
233 5% FBS was placed in each well.

234 In all cases, after 24 h, the protein-DNA gel particles or the protein-containing medium were
235 removed and 100 µL of MTT in PBS (5 mg mL-1) diluted 1:10 in medium without FBS and phenol
236 red was then added to the cells. The plates were incubated for a further 3 h, after which the
237 medium was removed, and the cells were washed once in PBS. Thereafter, 100 µL of DMSO was
238 added to each well to dissolve the purple formazan product. After 10 min on a microtitre-plate

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239 shaker at room temperature, absorbance of the resulting solutions was measured at 550 nm using
240 a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as a percentage of
241 cell viability inhibition against the respective controls.

242 2.5. Internalisation of AO-labeled PS-DNA gel particles

243 2.5.1. Preparation of AO-labeled PS-DNA gel particle dispersion

244 AO stock solution (10 µg mL^{-1}) was diluted 1:5 in a solution of DNA (2.5 mM). The AO-labeled PS-
245 DNA gel particle dispersion was prepared by nebulisation of the AO-labeled DNA into a solution of
246 protein (2.5 mM). After formation, the particle dispersion was filtered by a centrifugal concentrator
247 (cut-off 3,000 MWCO, Vivaspin 2) to remove the excess of free PS and non-incorporated AO.

248 2.5.2. Cellular uptake experiments

249 HeLa cells (5×10^4 cells mL^{-1}) were seeded into 24-well tissue plates on Corning´s circular glass
250 cover-slips at 37ºC and 5% CO_2 atmosphere. After incubation for 24h, the purified AO-labeled PS-
251 DNA gel particle dispersion was diluted 1:1 in medium without FBS and phenol red and then added
252 to the cells. Untreated cells stained with AO (1.5 µg mL^{-1}) in DMEM medium without FBS and
253 phenol red were used as a cell control. The plates were incubated for a further 2 h at 37ºC and 5%
254 CO_2 atmosphere, after which the medium was removed, and the cells were washed four times with
255 sterile PBS. After the final wash, the cells were fixed with 4% (v/v) paraformaldehyde in PBS for
256 15 min at room temperature and were washed twice with PBS.

257 Individual cover-slips were then mounted on clean glass slides with Prolong® Gold antifade
258 reagent (Invitrogen). Both contrast and fluorescence images were acquired with a Olympus BX41
259 microscope equipped with a UV-mercury lamp (100W Ushio Olympus) and a filter set type MNIBA3
260 (470-495 nm excitation and 505 nm dichromatic mirror) Images were digitised on a computer
261 through a video camera (Olympus digital camera XC50) and were analysed with an image
262 processor (Cell B analysis).

263 3. Results

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264 ## 3.1. Haemolytic assessments

265 ### 3.1.1. Haemolysis induced by proteins in solution

266 The haemolytic activity of both proteins at different pH was determined according to concentration.
267 In these experiments, haemolysis was determined at a fixed time (after 10 min of incubation) in the
268 presence of protein concentrations in the range of 100 to 2000 μg mL^{-1}. The haemolysis assay
269 showed that both proteins were non-haemolytic in nature. The haemolytic potential of a material is
270 defined as the measure of the extent of haemolysis that may be caused by the system when it
271 comes into contact with blood. At pH 7.4, both proteins were found to be non-haemolytic, the
272 extent of haemolysis being lower than the permissible level of 5% (Rao et Sharma, 1997; Lv et al.,
273 2007; He et al., 2009; Verkatesan et al., 2011). For the lowest concentration, the maximum
274 haemolysis values were 0.3% and 0.8% for the LS and PS proteins, respectively. At the highest
275 concentration, the value increased to 0.5% in the case of LS, and 1.7% in the case of PS. In both
276 cases, haemolysis was negligible at the highest concentration assayed (2000 μg mL^{-1}).
277 No significant differences were observed in the haemolytic response when varying the pH of the
278 buffer solution from 7.4 to 5.4. The isoelectric point (pI) value of the LS and PS proteins was
279 almost equal to 11 and 12, respectively, conserving their cationic character under the studied pH
280 interval. Although results on pH dependence of LS net charges demonstrated that the charges
281 varied between +8.5 at pH 5.5 to +7 at pH 8 (Desfougères, et al., 2010), there was no strong
282 alteration in the haemolytic responses. To the author's knowledge, nothing has been reported on
283 pH dependence of PS net charges.

284 The observed behaviour differed strongly from that observed, for instance, for surfactants in
285 solution (Morán et al., 2012). In this kind of system, haemolysis varied with the surfactant
286 concentration in a sigmoidal manner, until reaching total haemolysis.

287 ### 3.1.2 Haemolysis induced by protein-DNA gel particles

288 #### 3.1.2.1. Protein-DNA gel particles prepared by the dropwise addition method

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289 In order to evaluate the effect of protein-DNA complexation on the corresponding haemolytic
290 response, both the corresponding protein-DNA gel particles as well as the protein solutions at the
291 conditions used to prepare the protein-DNA gel particles, were incubated with the erythrocyte
292 suspensions for different lengths of time (ranging from 10 to 360 min) (Fig. 1). Using this approach,
293 the haemolytic response to the protein solutions corresponding to the conditions used to prepare
294 the protein-DNA gel particles, prior to interaction with the DNA drops, would provide us with
295 information about the maximum haemolytic response expected for these conditions. As regards the
296 different incubation times, it was expected that the haemolytic response to the protein solutions
297 would not alter over time. However, long incubation periods could affect the stability of the protein-
298 DNA gel particles, promoting the release of the protein into the solution and altering the haemolytic
299 response.

300 **[Fig. 1 here]**

301 Studies of the different protein systems in solution demonstrated that the haemolytic response was
302 weakly dependent on the protein composition in which particle formation took place (Fig. 1A). The
303 obtained profiles showed haemolytic percentages that remained constant between 10 and 120
304 min, increasing by only 5% by the end of the experiment (360 min). Note that the protein
305 concentrations assayed in these studies (60 mM, concentrations ranged between 3x 10^4 and 2x
306 10^5 μg mL^{-1} in the case of pure protein systems) were well above the 2x10^3 μg mL^{-1} assayed in the
307 case of pure protein systems in solution (see above). Our results demonstrated that both of the
308 pure proteins, LS and PS, as well as the mixtures at the imposed compositions (see Table 1) were
309 non-haemolytic in nature.

310 Individual protein-DNA gel particles incubated in the erythrocyte dispersion for periods of time
311 ranging between 10 and 360 min demonstrated no time-dependence in the haemolysis response.
312 In all cases, the percentages of haemolysis were lower than 3% (Fig. 1B).

313 **Relationship between the degree of haemolysis, number of erythrocytes and agglutination**
314 **of erythrocytes**

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315 Determination of haemolytic properties is one of the most common tests in studies of particle
316 interaction with blood components. Interpreting the results of these studies is complicated due to
317 variability in experimental approaches and a lack of universally accepted criteria for determining
318 the test-result validity. Most *in vitro* studies of particle-induced haemolysis evaluate the percentage
319 of haemolysis by spectrophotometrically detecting plasma-free haemoglobin derivatives after
320 incubating the particles with blood and then separating undamaged cells by centrifugation.
321 However, some particle interference due to haemoglobin precipitates adsorbed with the particles
322 on centrifugation has been reported, yielding a false negative result (Dobrovolskaia et al., 2008).

323 To avoid these false negative results in the haemolytic response of these protein-DNA particles,
324 the evolution of both haemolytic activity and the number of erythrocytes in the dispersions for each
325 time were determined simultaneously. Fig. 2A shows the results. There is a good relationship
326 between the degree of haemolysis found and the number of erythrocytes. Low values of
327 haemolytic responses correspond to a high number of erythrocytes in the corresponding
328 dispersion; and an increase in haemolysis corresponds to a decrease in the number of
329 erythrocytes. Thus, the effect of adsorbed haemoglobin on the particles can be considered
330 negligible.

331 There is, however, another point to be note. Starting for systems containing equimolar amount of
332 both proteins or higher PS content, a strong decrease of the number of erythrocytes was observed,
333 even for the shortest incubation time. This decrease, corresponding to a half of the initial number of
334 erythrocytes was independent of the haemolytic responses. This fact can be correlated with the
335 agglutination of the erythrocytes, which became visible for the erythrocyte dispersions incubated
336 with these systems (Fig. 2B).

337 **[Fig. 2 here]**

338

339 **Relationship between the DNA released and the degree of haemolysis**

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340 Previous work in our lab has demonstrated the potential application of these protein-DNA gel
341 particles in the controlled encapsulation and release of DNA (Morán et al., 2009a). The magnitude
342 of the DNA release was controlled and controlled release systems were achieved by changing the
343 LS/PS ratio in the protein solution where particles were formed.

344 Nevertheless, it was of major interest in this study to characterise these DNA gel particles,
345 considering their kinetics of DNA release and the induced haemolytic response simultaneously.
346 The release profiles of DNA from the protein-DNA gel particles in PBS buffer (pH 7.4) prepared
347 with different protein compositions were monitored simultaneously with the haemolysis response
348 induced by these protein-DNA gel particles in the same buffer solution.

349 The values for both the amount of DNA release and the percentage of haemolysis for each
350 composition after 360 min of incubation have been plotted in Fig. 3. It can be seen that the amount
351 of DNA released, for an almost constant haemolytic response, was strongly dependent on the
352 protein composition in which particle formation took place. In the range of LS/PS studied, the
353 amount of DNA that was released varied from 100 μg mL^{-1} in the case of the pure LS system to 3
354 μg mL^{-1} in the systems containing the PS protein. Further studies demonstrated that intermediate
355 compositions between the pure LS system and the LSPS15 system promoted a more progressive
356 decrease in DNA release with a similar haemolytic response (Fig. 3B).

357 **[Fig. 3 here]**

358

359 **Determination of protein content and complexation stoichiometries**

360 The observed differences in DNA release can be explained by differences in protein-DNA
361 complexation in these systems. To this end, the amount of protein released in the media by the
362 end of the experiment was determined (Fig. 4, black bars). The protein:DNA ratio in the particles
363 was determined from the protein released by the particles and the amount of DNA released into
364 the media (see Fig. 3 line plots and Fig. 4, grey bars). Fig. 4A shows the evolution of the
365 protein:DNA ratio according to protein composition.

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366 This protein-DNA ratio increased strongly from the pure LS system to the pure PS system. This
367 different distribution can be correlated with differences in the gelation process during particle
368 formation. It was expected that a homogeneous gelation would give rise to homogeneous
369 structures (solid particle) whereas a more inhomogeneous gelation process would form core-shell
370 particles (Morán et al., 2010).

371 In the present study, the stoichiometries obtained corroborated that the presence of PS favoured
372 the formation of solid particles. This model was supported by visual inspection, in which translucent
373 and opaque/condensed particles were obtained (Fig. 4B).

374 **[Fig. 4 here]**

375 **3.1.2.2. Protein-DNA gel particles prepared by the nebulisation method**

376 In previous studies in our lab, we have prepared nano-/micro-sized DNA gel particles by
377 nebulisation of DNA solutions (either single- (ssDNA) or double-stranded (dsDNA)) into an
378 oppositely charged surfactant or protein solution (Morán et al., 2009b). The size and size
379 distribution of the particle populations were investigated by means of fluorescence microscopy
380 (FM), photon correlation spectroscopy (PCS) and scanning electron microscopy (SEM). FM studies
381 suggest that the formation of the particles was carried out with conservation of the secondary
382 structure of the nucleic acid molecules. SEM on freeze-dried and Au-shadowed samples showed a
383 distribution of virtually spherical particles. Although the particle suspensions were investigated
384 without further purification it was found that, in addition to the size of the initial DNA droplets, the
385 cationic agent is a controlling parameter of the particle size. LS-DNA gel particles showed
386 diameters around 10 μm whereas the size of PS-DNA was around 400 nm.

387

388 In the present study, protein-DNA gel particles from mixed protein systems have been prepared by
389 the nebulisation method for the first time. The assayed conditions correspond to a half of the
390 previous studied concentrations (Morán et al., 2009b). Mixed protein-DNA particles prepared with
391 proteins with very different molecular weight (LS with a molecular mass of 14.3 kDa and PS with a

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392 molecular mass of 5.1 kDa) made particularly difficult to use centrifugal concentrators with a
393 suitable cut-off to efficiently remove both proteins. For this reason, these particle dispersions have
394 been studied without further purification.

395 The haemolytic activity of the protein-DNA gel particles prepared by the nebulisation method was
396 determined. For thus, several volumes (100-300 µL) of the dispersions containing the protein-DNA
397 gel particles were incubated in the erythrocyte dispersion for periods of time ranging between 10
398 and 360 min and haemolysis was determined. The obtained results demonstrated no time-
399 dependence in the haemolysis response. In all cases, the percentages of haemolysis were lower
400 than 1% (data not shown).

401 The protein concentrations assayed in these studies (2.5 mM, concentrations ranged between 3x
402 10^3 and 2x 10^4 µg mL^{-1} in the case of pure protein systems) were one order of magnitude lower
403 than those assayed in the case of protein-DNA gel particles obtained by the dropwise addition
404 method, where haemolytic responses were always lower than 3% (see Fig. 1B).

405
406 **3.2. Cytotoxic assessments**

407 Cytotoxicity plays a critical role in the efficiency of the delivery vectors. In order to deliver the DNA
408 into the cells, the cationic particles bind to the cell surface by electrostatic interaction, promote
409 endocytosis and release the genetic material inside the cell. Unfortunately, while high
410 concentrations of the delivery agents imply an increased chance of the DNA penetrating the cell
411 nucleus, they can also interfere with physiological processes within the cell, inducing cell death.
412 Thus, present research is aimed at designing gene delivery agents that are able to deliver DNA
413 into the cells with minimal toxicity (Rao et al., 2007).

414 **3.2.1.Cytotoxicy induced by proteins in solution**

415 Assessing the capacity of live cells to metabolise a tetrazolium colourless salt to a blue formazan
416 (MTT assay) is one of the most common methods used to perform indirect measurements of cell
417 viability. Dose–response curves for each protein, determined by MTT assays using tumour cell line

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418 HeLa and non-tumour cell line 3T3 fibroblasts, are given in Fig. 5. The cytotoxicity assays were
419 performed in the concentration range 50 and 2000 μg mL^{-1}. Although it is thought that proteins are
420 biocompatible and nontoxic compounds, our results have revealed that, as with other cationic
421 derivatives, LS and PS displayed concentration-dependent toxicity towards cells *in vitro*. LS
422 showed low cytotoxicity towards 3T3 cells, which displayed viability in the range 81% to 100% as
423 determined by the MTT assay (Fig. 5A) at the tested protein concentration range. In the case of
424 PS, viability changed from 7% to 100% according to the concentration. The corresponding IC_{50}
425 values were 140 μg mL^{-1} and >2000 μg mL^{-1} for PS and LS, respectively.

426 Analogous studies were carried out with the tumour cell line HeLa. The response to these two
427 proteins was very similar to that of the 3T3 cell line, as can be seen in Fig. 5B. The corresponding
428 IC_{50} values were 250 μg mL^{-1} and >2000 μg mL^{-1} for PS and LS, respectively.

429 **[Fig. 5 here]**

430 **3.2.2. Cytotoxicity *in vitro* induced by protein-DNA gel particles**

431 **3.2.2.1. Protein-DNA gel particles prepared by the dropwise addition method**

432 It was of great interest in this study to characterise these DNA gel particles considering the
433 cytotoxic response they induced. Fig. 6 shows the cytotoxic response of 3T3 and HeLa cell lines
434 treated with both the protein-DNA gel particles and the corresponding protein solutions, determined
435 by MTT assay. Cell viabilities of up to 80% were observed in almost all compositions when the
436 cytotoxicity of the corresponding protein-DNA gel particles was determined in both cell lines (Fig.
437 6A). However, relative viabilities were always lower than 10% when 3T3 cells were incubated in
438 the presence of pure and mixed protein systems. Similar results were obtained for the HeLa cell
439 line. In this case, cell viabilities increased step-wise to 20% (Fig. 5B).

440 **[Fig. 6 here]**

441

442 **3.2.2.2. Protein-DNA gel particles prepared by the nebulisation method**

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443 Although the molecular details of the mechanism by which cationic carriers mediate DNA delivery
444 are still poorly understood, current evidence supports the hypothesis that cationic lipid-DNA
445 complexes enter cells by means of endocytosis. Often, the particle size ranges from 100 nm to
446 over 1 µM, and evidently, the efficiency of cellular uptake and subsequent intracellular processing,
447 may depend on particle size (Rejman et al., 2004).
448 Using the MTT assay, the cytotoxic effect of these systems was determined. Fig. 7 shows the
449 relative cytotoxicity of the protein-DNA gel particles and the corresponding protein solutions
450 towards the 3T3 and HeLa cell lines. In both cases, the assayed concentration was 1.25 mM,
451 expressed in terms of protein concentration. Note that the LSPS7.5 composition has been also
452 included; considering the results obtained in the haemolysis *vs.* DNA release section (see Fig. 3B).

453 From Fig. 7A it can be deduced that 3T3 cells exhibited cell viabilities that can be modulated from
454 10 to 70% according to the protein composition in the protein-DNA gel particles. It is interesting to
455 note that the presence of a small quantity of PS produced a marked improvement in cell viability
456 compared to the pure LS system, and a small proportion of LS in the PS system produced a
457 marked reduction in the cell viability obtained in comparison with pure PS. A parallel trend was
458 observed when the cytotoxicity of these systems was assayed with the HeLa cell line. However,
459 relative viabilities of the 3T3 cell line were always higher than those observed in the case of the
460 HeLa cell line, and the tumour cell line HeLa appeared to be more sensitive to the deleterious
461 effects of the protein-based particles than 3T3 fibroblasts.

462 In order to compare, the relative viabilities obtained with the corresponding protein solutions have
463 also been plotted (Fig. 7B). Cell viability in the 3T3 cell line was strongly dependent on the protein
464 composition, ranging from a maximum value of around 80% with pure LS to minimum values of
465 around 10% for systems containing the PS protein.

466 The observed differences in cytotoxicity may be correlated with differences in protein-DNA
467 complexation in these systems. Consequently, we determined the initial amount of protein in the
468 media, as well as the amount of protein remaining in the dispersion containing the protein-DNA gel

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469 particles formed by the nebulisation method. From these values, the degree of complexation in the
470 different systems was determined. Fig. 7C shows the evolution of the degree of complexation
471 according to the LS/PS ratio. In the case of the protein-DNA gel particles, it was expected that the
472 higher the degree of complexation, the smaller the amount of protein that would be released in
473 solution, an amount which would be able to interact with the cells and reduce their viability.
474 Independently of the cell line response, the differences in the degree of complexation were in
475 agreement with the observed trend in cell viabilities (Fig. 7D).

476 **[Fig. 7 here]**

477 **Relative viabilities of tumour and non-tumour cell lines**

478 Further data on the concentration-dependent cytotoxicity of these small-sized protein-DNA gel
479 particles was obtained by examination of the relative cytotoxicity of several dilutions of the
480 corresponding dispersions (Fig. 8). Note that the concentrations are expressed in terms of protein
481 concentration, given in mM. The highest concentration corresponded to the conditions shown in
482 Fig. 7A. By fitting the curves in Fig. 8, it was possible to determine the IC_{50} of these protein-based
483 DNA carriers (Table 2).

484 **[Fig. 8 here]**

485 **[Table 2 here]**

486
487 It can be seen from the IC_{50}-values that the two cells lines showed markedly different sensitivity to
488 the cytotoxic effects of these small-sized protein-DNA particles. Except in the case of the pure
489 systems, which displayed identical cytotoxicity in both cell lines, the tumour cell line HeLa
490 appeared to be more sensitive to the deleterious effects of the mixed protein-based particles than
491 3T3 fibroblasts (significant differences between 3T3 and HeLa for the same conditions are
492 indicated in Table 2 with an asterisk).

493 **Internalisation of AO-labeled PS-DNA gel particles**

494 The ability of these particles to effectively cross biological barriers, which would allow their use in
495 the delivery of DNA, was evaluated. PS-DNA gel particles, which showed both suitable particle

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496 size and minimum cytotoxic responses, were chosen for this kind of experiments. For this purpose,
497 AO-labeled PS-DNA gel particles were prepared and the particle uptake by HeLa cells was
498 assayed. In general, particles exposed to cells in the absence of serum have a stronger adhesion
499 to the cell membrane and higher internalization efficiency, in comparison to what is observed in
500 medium containing serum, when a preformed protein layer is present on their surface (Lesniak et
501 al., 2012). In order to perform these studies on the most favourable conditions the experiments
502 were carried in absence of serum. Preliminary studies on cellular uptake of protein DNA gel
503 particles prepared with DNA solutions in the presence of the nucleic acid selective dye acridine
504 orange demonstrated that these particles could be internalised by HeLa cells (Fig. 9D).

505 **[Fig. 9 here]**

506

507 ## 4. Discussion

508 A general understanding of the interaction of DNA with oppositely charged amphiphiles and
509 polyelectrolytes provides a basis for developing novel DNA gel particles, including protein-DNA gel
510 particles. When used as DNA carriers, understanding the interactions of these DNA gel particles
511 with red blood cells and established cells *in vitro* is crucial for improving their behaviour *in vivo*. To
512 this end, we examined this interaction by using erythrocytes as a model of a biological membrane
513 system. Firstly, the haemolytic activity of these two proteins in solution was studied according to
514 their concentration and the concentration-dependent curves were determined. It may be concluded
515 that both proteins are non-haemolysing agents at the highest concentration assayed
516 (2000 μg mL^{-1}).

517 One drawback of protein-DNA gel particles, in toxicological terms, is the need for a cationic protein,
518 which may cause some cell damage. Our results demonstrated that both of the pure proteins, LS
519 and PS, as well as the mixtures at the imposed compositions (see Table 1) at which particles were
520 prepared, were non-haemolytic in nature. Studies of individual protein-DNA gel particles incubated
521 in the erythrocyte dispersion over periods of time ranging between 10 and 360 min demonstrated

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522 no time-dependence in the haemolysis response (Fig. 1). In all cases, the percentages of
523 haemolysis were lower than the permissible 5% ((Rao et Sharma, 1997; Lv et al., 2007; He et al.,
524 2009; Verkatesan et al., 2011). Similar results were obtained with protein-DNA gel particles
525 obtained by the nebulisation method. Consequently, the *in vitro* haemolysis results suggest that
526 these protein-DNA gel particles show promise as long-term blood-contacting medical devices for
527 several applications.

528 The agglutination induced by the protein-DNA gel particles containing equimolar amount of both
529 proteins or higher PS content (Fig. 2) may be caused by increased binding of the particles to the
530 erythrocyte membrane, which might deformed cells and hence decrease the repulsion among them
531 (Li et al., 2008). This behaviour could be attributed to differences in the binding characteristics of
532 these two proteins, with different total charge and linear charge density: LS is a globular protein
533 that has a net charge of + 9 at neutral pH, whereas PS is a highly positively charged linear protein
534 with an overall charge of +21.

535 It was of major interest in this investigation to characterise these DNA gel particles considering
536 their kinetics of DNA release and the induced haemolytic response simultaneously (Fig. 3). It was
537 observed that the amount of DNA released, for an almost constant haemolytic response, was
538 strongly dependent on the protein composition at which particle formation took place. In the range
539 of LS/PS studied, the amount of DNA that was released varied considerably, from 100 μg mL^{-1} in
540 the case of the pure LS system to almost 3 μg mL^{-1} in the presence of the PS protein. Achieving
541 high incorporation efficiencies and control over release kinetics present significant challenges in
542 the development of a novel nonviral vector for gene therapy. Previous studies (Morán et al., 2009a)
543 demonstrated, however, that LS-DNA particles exhibited initial fast burst release behaviour through
544 a dissolution mechanism, without protecting the DNA secondary structure. However, the
545 magnitude of DNA release was controlled and controlled release systems were achieved by
546 changing the LS/PS ratio in the protein solution where particles were formed. The observed
547 differences in DNA release can be explained by differences in protein-DNA complexation in these
548 systems. As shown in Fig. 4, the protein-DNA ratio increased strongly from the pure LS system to

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549 the pure PS system. The stoichiometries obtained would appear to confirm that the presence of PS
550 favours the formation of solid particles.

551 Although proteins are thought to be biocompatible and nontoxic-compounds, the results of our
552 safety evaluation with the established 3T3 and HeLa cell lines indicated that, as with other cationic
553 derivatives, LS and PS displayed concentration-dependent toxicity towards cells *in vitro* (Fig. 5). In
554 these experiments, cytotoxicity was determined in the presence of a range of protein
555 concentrations, enabling us to define the protein concentration required to inhibit cell growth by
556 50% compared with an untreated control (IC_{50}). In the case of LS, its IC_{50} was found to be higher
557 than 2000 μg mL^{-1} in both cell lines. For PS, it was found to be 140 and 250 μg mL^{-1} for 3T3 and
558 HeLa cell lines, respectively. These differences could be attributed to differences in the binding
559 characteristics of these two proteins, with different total charge and linear charge density: LS is a
560 globular protein that has a net charge of + 9 at neutral pH, whereas PS is a highly positively
561 charged linear protein with an overall charge of +21.

562 Cell culture studies have greatly increased the understanding of cellular functions and complex
563 signalling pathways and have been routinely used for toxicity screening of new compounds. All cell
564 culture studies hinge on the capacity to maintain a suitable cell culture environment. However,
565 these protocols may be altered by the presence of particles. Previous studies in our laboratory
566 have verified the absorption of the culture media by the particles when 3T3 and Hela cell lines
567 were incubated in the presence of some surfactant-DNA gel particles prepared by the dropwise
568 addition method. In this case, the obtained cell viabilities were close to 5% (results not published).
569 Although the IC_{50} values of the corresponding surfactants in solution were not very high (with
570 values around 10 μg mL^{-1}), this low cell viability may be correlated with the physicochemical
571 properties of these DNA gel particles. Their swelling behaviour could restrict the optimal nutrition
572 and physiological parameters to support the growth of cells.

573 In was of great interest in this study to characterise these protein-DNA gel particles considering the
574 cytotoxic response they induced. Visual inspection of the corresponding plates exhibited no evident

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575 changes on the volume and characteristics of the culture media when 3T3 and HeLa were
576 incubated in the presence of individual protein-DNA gel particles during 24h. Although these
577 particles are several magnitudes larger than cells and cannot be internalized as a whole, this study
578 demonstrates that the physicochemical properties of these protein-DNA gel particles may not affect
579 their cytotoxic characterization under standard protocols. These protein-DNA gel particles showed
580 cell viabilities higher than 80% in almost all cases, except in the case of cells incubated in the
581 presence of LSPS85-DNA gel particles (Fig. 6A). These results can be correlated with differences
582 in protein content on these particles, as have been evaluated using the method of Bradford (Fig.
583 4A). Although the protein-DNA gel particles remain visible on the plates after 24h of incubation, the
584 total protein content on LSPS85-DNA gel particles (413 μg mL^{-1} of protein) in comparison with
585 comparing with the other systems (amount ranged between 37 and 288 μg mL^{-1} of protein) might
586 explain these differences in protein-induced cytotoxicity from protein-DNA gel particles. Significant
587 differences between the cell viability obtained in the presence of protein-DNA gel particles and the
588 corresponding proteins solutions have been obtained (Fig. 6B).

589 In this study, small-sized mixed protein-DNA gel particles were prepared for the first time, and their
590 cytotoxicity was evaluated. Although the particle dispersions were studied without further
591 purification, and both free protein and protein-DNA gel particles were present in the obtained
592 dispersions, the cytotoxic responses shown in Fig. 7A were observed to be significantly different to
593 that observed with proteins in solution (Fig. 7B).

594 This behaviour can be correlated with the capacity of the different protein systems to form weaker
595 or stronger protein-DNA complexes. In the case of the protein-DNA gel particles, it was expected
596 that the higher the degree of complexation, the smaller the amount of protein that would be
597 released in solution, an amount which would be able to interact with the cells and reduce their
598 viability. Determination of the degree of complexation in the different systems showed that it was
599 strongly dependent on the protein composition at which particle formation took place (Fig. 7C).
600 Independently of the cell line, the observed trend in cell viabilities was in agreement with the
601 observed degree of complexation (Fig. 7D).

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602 The observed differences in cytotoxicity between protein-DNA particles prepared by the dropwise
603 addition method and the nebulisation method could be related to differences in the kinetics of
604 dissolution/release profiles. Studies of DNA release from protein-DNA particles formed by the
605 dropwise addition method have demonstrated that these particles can present DNA release
606 profiles of up to 1000 h, confirming the stability of these protein-DNA gel particles (Morán et al.,
607 2009a). In the present study, the stability of these particles in the culture medium was also
608 confirmed. Supported by visual observation, the particles remained present in the well plate after
609 24h of incubation. This behaviour and the fact that the observed cytotoxicity was almost
610 independent of the protein composition (see Fig. 6A) corroborate this argument. Although the
611 profiles of dissolution/release of the protein-DNA gel particles prepared by the nebulisation method
612 have not yet been determined, it is expected that smaller particles will show faster dissolution
613 profiles. As a consequence of the protein release, a more composition-dependent cytotoxic
614 response compared with that observed at large-sized particles could be awaited. In the case of
615 these small-sized protein-DNA particles the cytotoxic responses were strongly dependent on the
616 protein composition (see Fig. 7A).

617 The preparation of the protein-DNA gel particles by the nebulisation method enabled us to obtain
618 particle dispersions in order to evaluate the effect of the concentration (in this study, expressed as
619 protein concentration). Observation of IC_{50}-values showed that the two cells lines were markedly
620 different in sensitivity to the cytotoxic effects of these protein-DNA particles (Fig. 8 and Table 2).
621 Except in the case of the pure systems, which displayed identical cytotoxicity in both cell lines, the
622 tumour cell line HeLa was more sensitive to the deleterious effects of the mixed protein-based
623 particles than 3T3 fibroblasts (significant differences between 3T3 and HeLa for the same
624 conditions are indicated in Table 2 with an asterisk). Consequently, the mixing procedure had a
625 clear modulating effect on the relative cytotoxicity of these systems towards tumour and non-
626 tumour cell lines.

627 The ability of protein-DNA gel particles to effectively cross biological barriers was evaluated. To
628 this end, PS-DNA particle dispersions were prepared by nebulisation of DNA containing the nucleic

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629 acid selective dye acridine orange. After formation, the particle dispersions were filtered by
630 centrifugal concentrators to remove the excess of protein as well as the non-incorporated dye.
631 Although using this procedure some leaking of the dye from the protein-DNA gel particle can be
632 observed, which may permeate through the cell membrane and associate with genomic DNA, to
633 fluoresce, clear differences between the stained HeLa cell controls and the cells exposed to AO-
634 labeled PS-DNA gel particles (Fig. 9B and 9D, respectively). These preliminary results indicate
635 cellular uptake and internalisation of these protein-DNA particles, a prerequisite for subsequent
636 DNA delivery.

637 5. Concluding remarks

638 Mixtures of two cationic proteins, lysozyme (LS) and protamine sulphate (PS) were used to
639 prepare protein-DNA gel particles employing associative phase separation and interfacial diffusion.
640 Protein-DNA gel particles prepared by both methods (dropwise addition and nebulisation) were
641 demonstrated to be promising long-term blood-contacting medical devices based on their
642 negligible haemolytic effect. Cytotoxicity studies demonstrated that the protein solutions at the
643 concentration at which particles were prepared induced poor viabilities in 3T3 and HeLa cell lines.
644 However, cytotoxicity towards 3T3 and HeLa cell lines was reduced significantly when
645 administered in the protein-DNA gel particles prepared by dropwise addition method, with cell
646 viabilities higher than 80%. In contrast, the cytotoxic responses induced by small-sized protein-
647 DNA gel particles were strongly dependent on the protein composition, with viabilities ranging
648 between 10% and 70%. Under these conditions, the tumour cell line HeLa was more sensitive to
649 the deleterious effects of the mixed protein-based particles than 3T3 fibroblasts. Preliminary results
650 indicate cellular uptake and internalisation of these protein-DNA particles, a prerequisite for
651 subsequent DNA delivery.

652 Several points are of interest in these protein-DNA gel particles: firstly, unlike delivery in an
653 aqueous solution, the cytotoxicity of the protein system can be reduced when the opposite charges
654 between proteins and DNA spontaneously result in complexation due to electrostatic interactions.

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655 Secondly, the magnitude of DNA entrapment can be controlled and controlled release systems
656 achieved through the formation of a DNA-protein complex network giving rise to these protein-DNA
657 gel particles. The decrease in cytotoxicity as well as the formation of a releasable high DNA
658 content reservoir renders these protein-DNA gel particles promising protein-based DNA vehicles
659 for use as a nonviral gene delivery system. Current research focuses on the preparation of protein-
660 DNA gel particles using plasmid DNA. Further research on the particle uptake mechanism and the
661 kinetics of drug uptake and retention in the different cell lines will be useful to establish the efficacy
662 of these protein-DNA gel particles for various therapeutic applications.

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666 **References**

667 Abbasi, S.; Paul, A.; Prakash, S. 2011. Cationic albumin nanoparticles for enhanced drug delivery
668 to treat breast cancer: preparation and in vitro assessment. Cell Biochem. Biophys., 61, 277-287.

669 Boverhof, D. R., David, R. M. 2010. Nanomaterial characterization: considerations and needs for
670 hazard assessment and safety evaluation, Anal. Bioanal. Chem. 396, 953–961.

671 Bradford, M. M. 1986. A rapid and sensitive method for the quantification of micrograms quantities
672 of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254.
673

674 Costa, D.; Morán, M. C.; Miguel, M. G.; Lindman, B. 2008. Cross-linked DNA Gels and Gel
675 Particles. In: R. S. Dias, B Lindman, editors. DNA Interactions with Polymers and Surfactants. New
676 Jersey: Wiley Interscience.Lasic, D. 1997. Liposomes in Gene delivery, CRC Press: Boca Raton,
677 FL.
678

679 Crespi, C. L. 1995. Xenobiotic-metabolizing human cells as tools for pharmacological and
680 toxicological research, Adv. Drug Res., 26, 179-235.
681

682 Davis, M. E. 2002. Non viral-gene delivery systems. Curr. Opin. Biotechnol. 13, 128-31.
683

Accepted Manuscript

ACCEPTED MANUSCRIPT

684 Desfougères, Y.; Croguennec, T.; Lechevalier, V.; Bouhallab, S.; Nau, F. 2010. Charge and size
685 drive spontaneous self-assembly of oppositely charged globular proteins into microspheres. J.
686 Phys. Chem. B.114, 4138-4144.
687

688 Dobrovolskaia, M.A.; Clogston, J. D.; Neun, B. W.; Hall, J. R. B.; Patri, A. K.; McNeil, S. E. 2008.
689 Method for analysis of nanoparticle hemolytic properties *in vitro*. Nano Lett., 8, 2180-2187.

690 Fröhlich, E., Meindi, C., Roblegg, E., Griesbacher, A., Pieber, T.R. 2012. Cytotoxity of
691 nanoparticles is influenced by size, proliferation and embryonic origin of the cells used for testing.
692 Nanotoxicology, 6, 424-439.

693 Hawkins, M. J.; Soon-Shiong, P.; Desai, N. 2008. Protein nanoparticles as drug carriers in clinical
694 medicine. Adv. Drug Delivery Rev., 60, 876-885.
695

696 He, M.; Zhao, Z.; Yin, L.; Tang, C.; Yin, C. 2009. Hyaluronic acid coated poly(butyl cyanoacrylate)
697 nanoparticles as anticancer drug carriers. Int. J. Pharm.373, 165-173.
698

699 Hillegass, J. M., Shukla, A., Lathrop, S. A., MacPherson, M. B., Fukagawa, N. K., Mossman, B.
700 T.2009. Assessing nanotoxicity in cells in vitro, WIREs Nanomed. Nanobiotechnol. 2, 219–231.
701

702 Jondeau, A.; Dahbi, L.; Bani-Estivals, M. H.; Chagnon, M.C. 2006. Evaluation of the sensitivity of
703 three sublethal cytotoxicity assays in human HepG2 cell line using water contaminants. Toxicology,
704 226, 218-228.
705

706 Kratz, F. 2008. Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles.
707 J. Controlled Release, 132, 171-183.

708

709 Lasic, D. 1997. *Liposomes in Gene delivery*, CRC Press: Boca Raton, FL.

710 Ledley, F. D.1996. Pharmaceutical approach to somatic gene therapy. Pharm. Res. 13, 1595-
711 1614.
712

713 Lesniak, A., Fenaroli, F., Monopoli, M. P., Aberg, C., Dawson, K.A., Salvati, A. 2012. Effects of
714 the presence or absence of a protein corona on silica nanoparticle uptake and impact on cells,
715 ACS Nano, 6, 5845–5857.
716

ACCEPTED MANUSCRIPT

717 Li, S-Q; Zhu, R-R.; Zhu, H, Xue, M; Sun, X-Y.; Yao, S-D; Wang, S-L. 2008. Nanotoxicity of TiO_2
718 nanoparticles to erythrocyte in vitro. Food Chem Toxicol, 46, 3623-3631.
719
720 Lindman, B.; Dias, R. S.; Miguel, M. G.; Morán, M. C. Costa, D. 2009. Manipulation of DNA by
721 Surfactants. In: D. Platikanov, D. Exerowa, editors. Highlights in Colloid Science. Wiley-VCH.
722
723 Lindman, B.; Dias, R. S.; Miguel, M. G.; Morán, M. C.; Costa, D. 2010. DNA-Surfactants Systems:
724 Particles, Gels and Nanostructures. In: Nanoscience: Colloidal and Interfacial Aspects. CRC Press
725 Taylor & Francis.
726
727 Lv, Y. H.; Chen, X. G.; Wang, Q. Z.; Wang, Y.; Zhang,J.; Liu, C. S.; Liu, C. G.; Meng, X. H.; Yu,
728 L. J. 2007. Synthesis and characterization of chitosan-based biomaterials modified with different
729 active groups and their relationship with cytotoxicity. Journal of Wuhan University of Technology-
730 Materials Science Edition, 22, 695-700.
731
732 Martinez, V.; Corsini, E.; Mitjans, M.; Pinazo, A.; Vinardell, M. P. 2006. Evaluation of eye and skin
733 irritation of argininge-derivative surfactants using different in vitro endpoints as alternatives to the in
734 vivo assays. Toxicol. Lett., 164, 259-267.
735
736 Morán, M. C.; Alonso, T.; Lima, F. S.; Vinardell, M. P.; Miguel, M. G.; Lindman, B., 2012. Counter-
737 ion effect on surfactant-DNA gel particles as controlled DNA delivery systems. Soft Matter, 8,
738 3200-3211.
739
740 Morán M. C.; Pais, A.A.C.C.; Ramalho, A.; Miguel, M. G.; Lindman, B. 2009a. Mixed protein
741 carriers for modulating DNA release. Langmuir, 25, 10263–10270.
742
743 Morán, M. C.; Baptista, F. R.; Ramalho, A.; Miguel, M. G.; Lindman, B. 2009b. DNA gel
744 nanoparticles: preparation and controlling the size. Soft Matter, 5, 2538-42.
745
746 Morán, M. C.; Miguel, M. G.; Lindman, B. 2010. DNA gel particles. Soft Matter, 6, 3143-3156.
747
748 Morán, M. C.; Mitjans, M.; Martínez, V.; Fortuño, J.; Botta, A.; Nogueira, D. R.; Vinardell, M. P. 2013.
749 Current directions in DNA gel particles In: D. Muñoz-Torrero, A. Cortés, E. L. Mariño, editors, Recent
750 Advances in Pharmaceutical Sciences III.

ACCEPTED MANUSCRIPT

751 Morán, M. C.;Miguel, M. G.; Lindman, B. 2007a. DNA gel particles: Particle preparation and
752 release characteristics. Langmuir, 23: 6478–81.
753
754 Morán, M.C.; Miguel, M. G.;Lindman, B. 2007b. Surfactant-DNA gel particles: Formation and
755 release characteristics. Biomacromolecules, 8, 3886–92.
756
757 Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to
758 proliferation and cytotoxicity assays. J. Immunol. Methods, 65, 55-63.
759
760 Pack, D. W.; Putnam, D.; Langer, R. 2000. Design of imidazole-containing endosomolytic
761 biopolymers for gene delivery. Biotechnol. Bioeng. 67, 217-23.
762
763 Rao, G.; Yadava, P.; Hughes. 2007. Rationally designed synthetic vectors for gene delivery, J. The
764 Open Drug Delivery Journal, 1, 7-19.
765
766 Rao, S. B.; Sharma, C. P. 1997. Use of chitosan as a biomaterial: Studies on its safety and
767 hemostatic potential. J. Biomedical Materials Research, 34, 21-28.
768
769 Rhaese, S.; Briesen, H. ; bsamen-Waigmann, H.; Kreuter, J.; Langer, K. 2003. Human serum
770 albumin-polyethylenimine nanoparticles for gene delivery. J. Controlled Release, 92, 199-208.
771
772 Rejman, J.; Oberle, V.; Zuhorn, I. S.; Hoekstra, D. 2004. Size-dependent internalization of particles
773 via the pathways of chathrin- and caveolae-mediated endocytosis. Biochem. J., 377, 159-69.
774
775 Robbens J., Vanparys C., Nobels I., Blust R., Hoecke K. V., Janssen C., Schamphelaere K. D.,
776 Roland K., Blanchard G., Silvestre F., Gillardin V., Kestemont P., Anthonissen R., Toussaint O.,
777 Vankoningsloo S., Saout C., Alfaro-Moreno E., Hoet P., Gonzalez L., Dubruel P., Troisfontaines
778 P.2010. Eco-, geno-, and human toxicology of bio-active nanoparticles for biomedical applications,
779 Toxicology, 269,170–181.

780
781 Saenger, W. 1984. Principles of nuclei structure. New York: Springer-Verlang.
782
783 Sambrook, J.; Fritsch, E. F.; Maniatis, E. F. 1989. Molecular Cloning: a laboratory manual. New
784 York: Cold Spring Harbor Laboratory Press.
785

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786 Venkatesan, P.; Puvvada, N.; Dash, R.; Prashanth Kumar, B. N.; Sarkar, D.; Azab, B.; Pathak, A., Kundu, S. C.; Fisher, P. B., Mandal, M. 2011. The potential of celecoxib-loaded hydroxyapatite-chitosan nanocomposite for the treatment of colon cancer. Biomaterials, 32, 3794-806.

790

791 **FIGURE CAPTIONS**

792

793 **Fig. 1**. Protein solutions (A) and protein-DNA gel particles (B) induced haemoglobin release from rat erythrocytes as a function of time.

795 **Fig. 2.** Time-course of the haemolytic response and the number of erythrocytes in the dispersions (A) and agglutination of rat erythrocytes observed by optical microscopy after 60 min of incubation with the protein-DNA gel particles (B).

798 **Fig. 3**. Protein-DNA particle-induced haemoglobin release from rat erythrocytes and DNA release after 360 min of incubation (A) and kinetics of DNA release profiles for the studied protein-DNA particles.

801 **Fig. 4.** Complexation stoichiometries of the protein-DNA gel particles according to the protein composition (A). Detailed images of the corresponding protein-DNA gel particles (B) (Adapted from Morán et al., 2009a).

804 **Fig. 5.** Concentration-dependent relative viabilities of 3T3 cells (A) and HeLa cells (B) treated with LS and PS for 24 h determined by MTT assay. The data correspond to the average of three independent experiments ± standard deviation.

807 **Fig. 6.** Relative viabilities of 3T3 and HeLa cells treated with individual protein-DNA gel particles (A) and the corresponding protein solutions (B) for 24 h, determined by MTT assay. The data correspond to the average of three independent experiments ± standard deviation. In all cases, significant differences ($p<0.05$) between 3T3 and HeLa for the same conditions were found. *Significantly different ($p<0.05$) from the corresponding protein solution.

812 **Fig. 7.** Relative viabilities of 3T3 and HeLa cell lines treated with the protein-DNA gel particles dispersion (A) and the corresponding protein solutions (B) for 24 h, determined by MTT assay. In both cases, the assayed concentration was 1.25 mM, expressed in terms of protein concentration. The data correspond to the average of three independent experiments ± standard deviation. * Significantly different ($p<0.05$) from the corresponding protein solution. (C) Complexation stoichiometries of the protein-DNA gel particles according to the protein composition. (D)

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818 Comparison between the complexation stoichiometries of the protein-DNA gel particles and the
819 relative viabilites of 3T3 and HeLa cell lines according to protein composition.

820 **Fig. 8.** Concentration-dependent relative viabilities of 3T3 (A) and HeLa cells (B) treated with the
821 protein-DNA gel particles prepared by the nebulisation method for 24 h, determined by MTT assay.
822 The data correspond to the average of three independent experiments ± standard deviation.

823 **Fig. 9.** Representative contrast microscopy images (A) and fluorescence images (B) of HeLa
824 control. Contrast microscopy (C) and fluorescence images (D) of HeLa cells incubated during 2h in
825 the presence of PS-DNA particles. 40X Magnification.

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Table 1. Composition (w/w) of the DNA carrier systems expressed as LS/PS ratio

System	LS/PS (w/w)
LS	100/0
LSPS15	15/85
LSPS30	30/70
LSPS50	50/50
LSPS70	70/30
LSPS85	85/15
PS	0/100

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Table 2. Cytotoxic properties of the protein-based DNA carrier systems prepared by the nebuliser method. Note that the IC_{50} values are given in terms of protein concentration (mM). *Significantly different at $p<0.05$

System	3T3 IC_{50} (mM)	HeLa IC_{50} (mM)
LS-DNA	< 0.16	< 0.16
LSPS7.5-DNA*	> 1.25	0.88
LSPS15-DNA	> 1.25	0.92
LSPS30-DNA*	> 1.25	0.32
LSPS50-DNA	> 1.25	0.92
LSPS70-DNA	0.80	0.60
LSPS85-DNA*	0.75	0.68
PS-DNA	> 1.25	> 1.25

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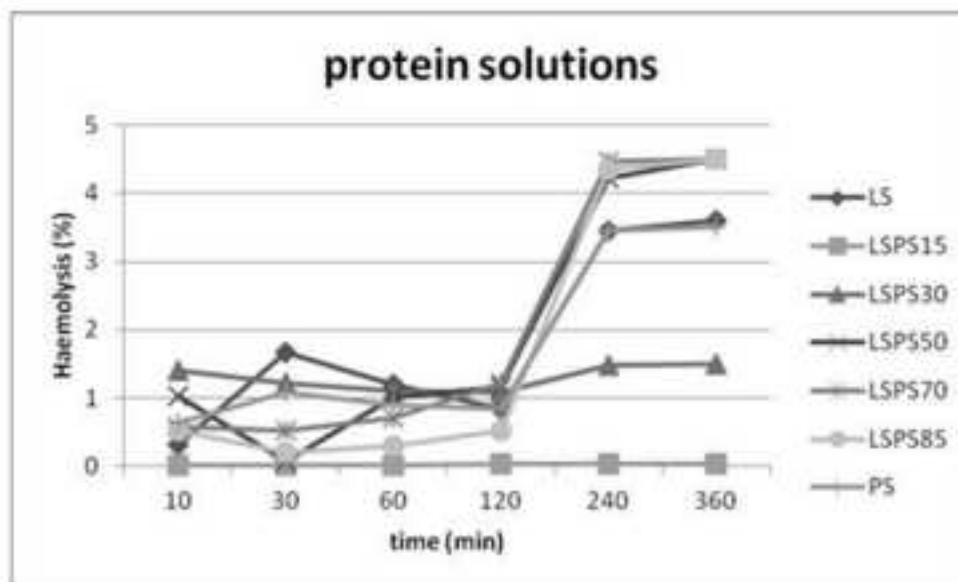
Figure 1



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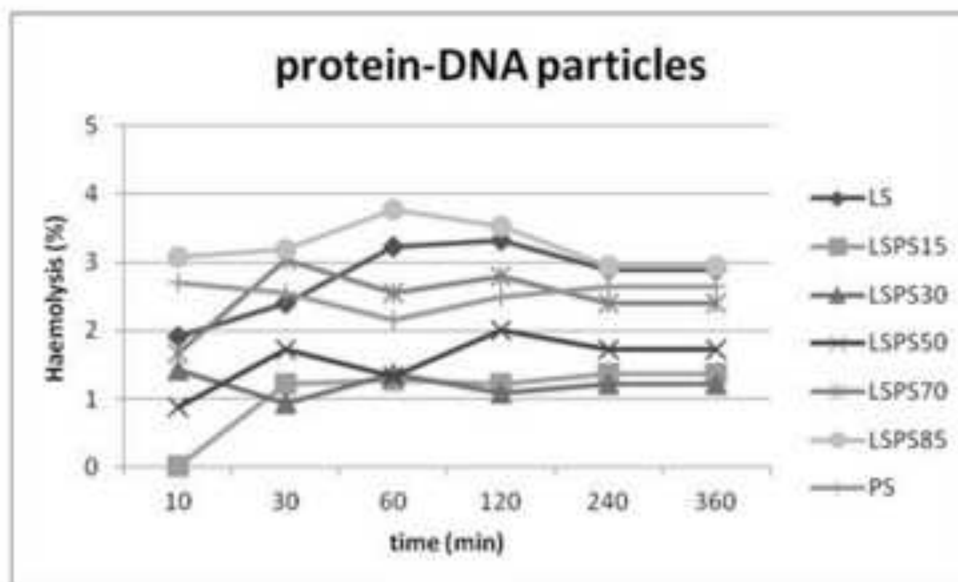

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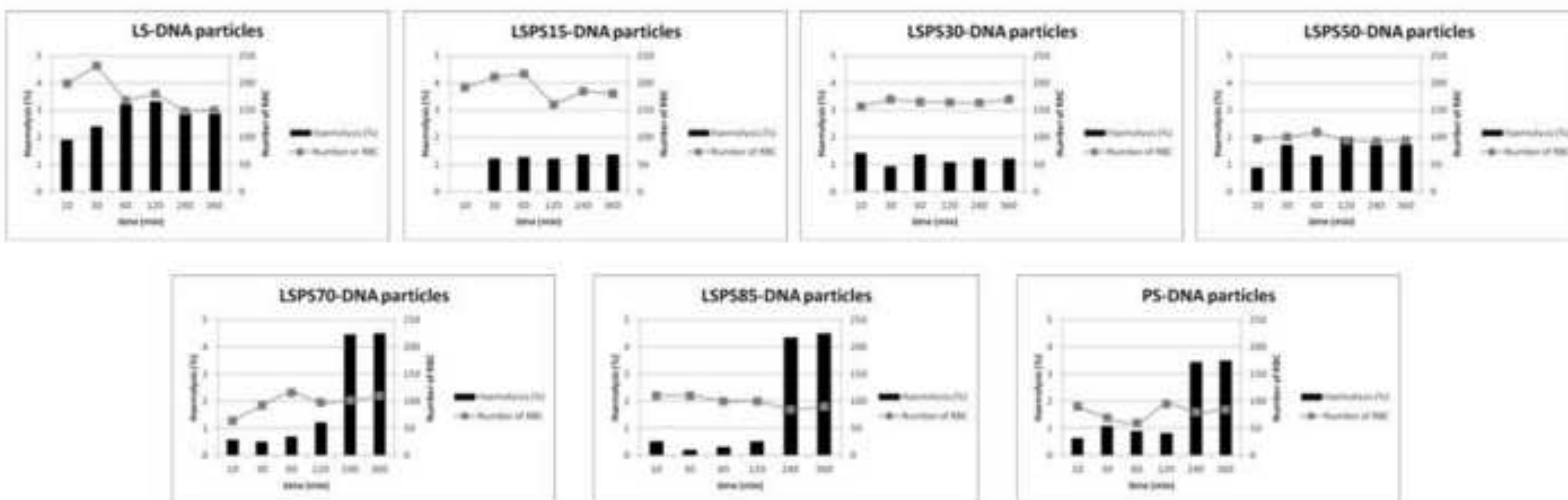

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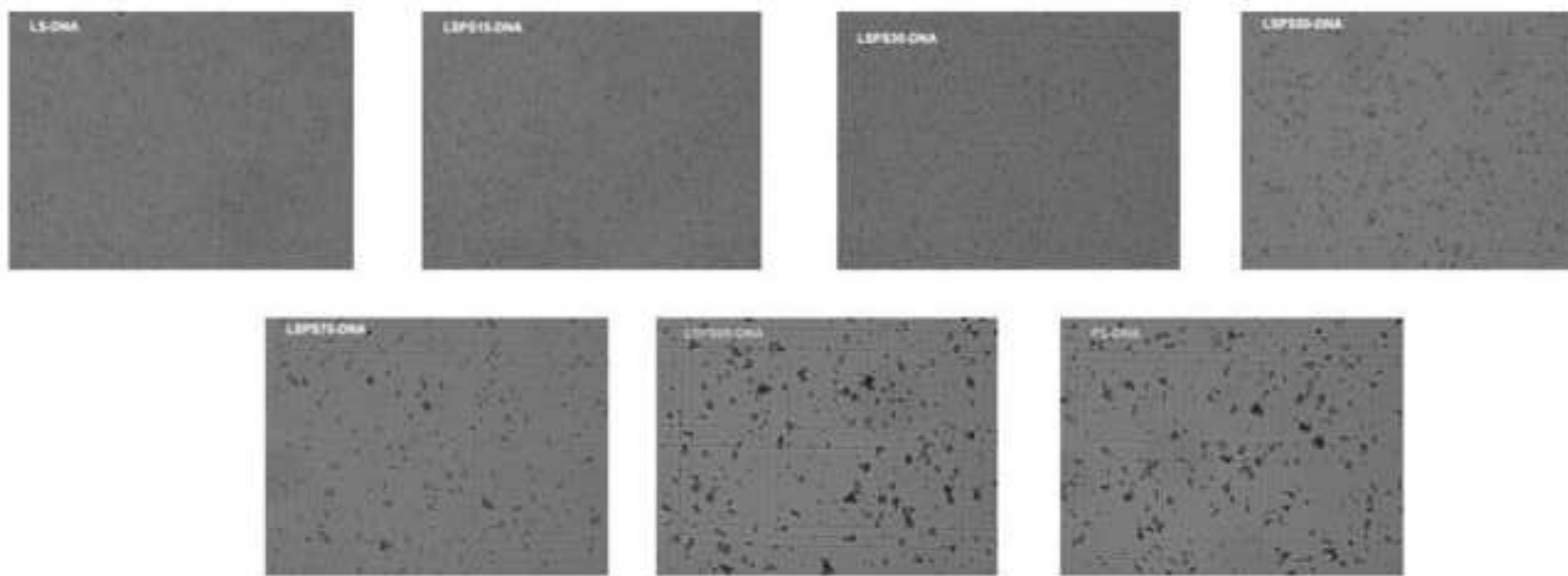

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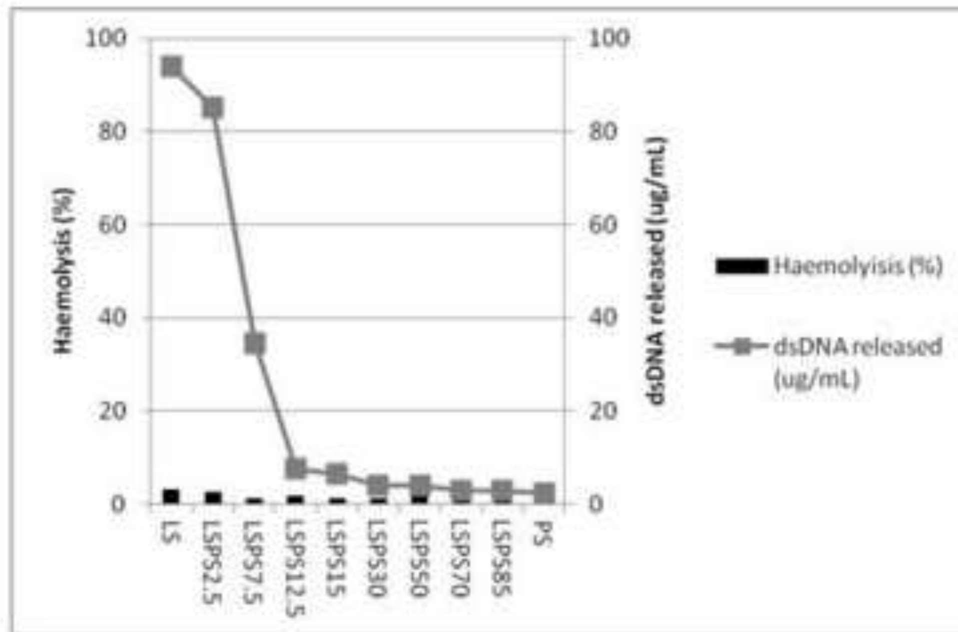

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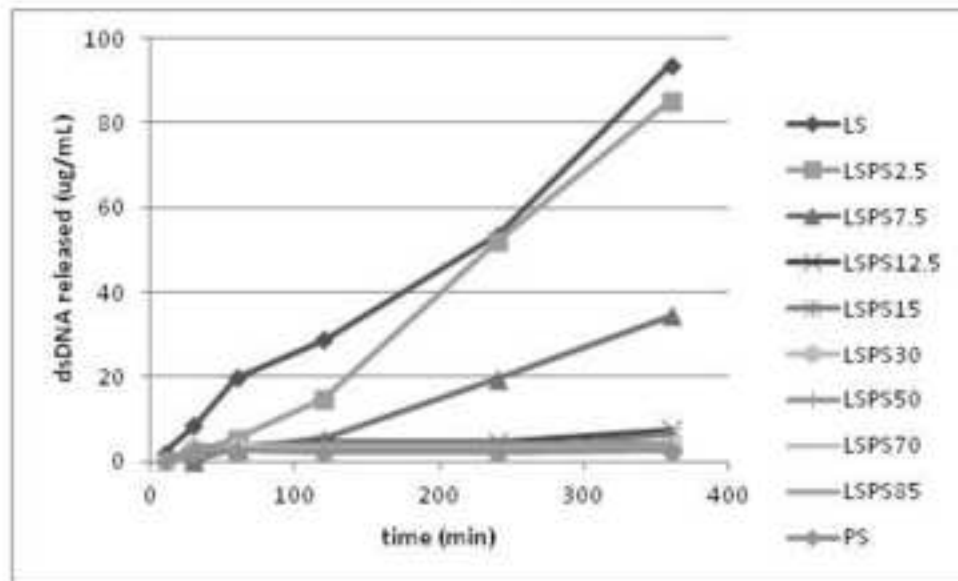

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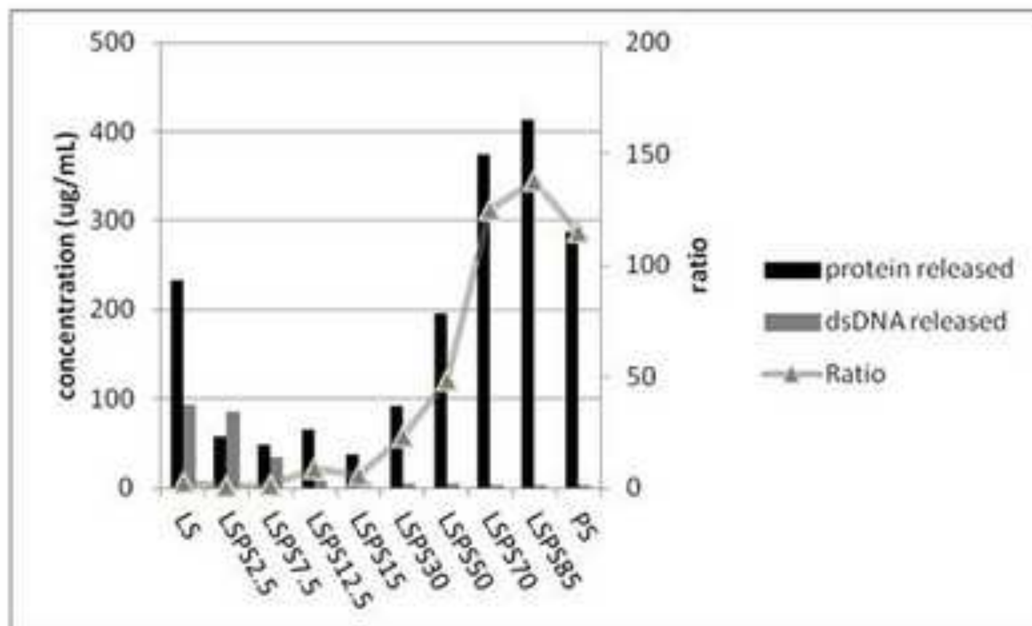

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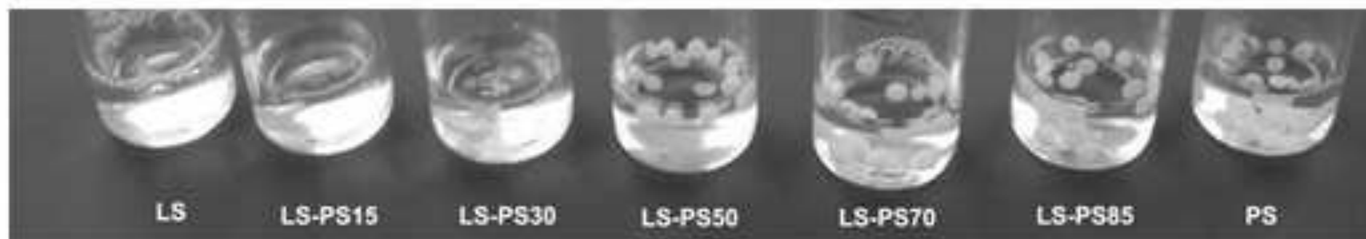

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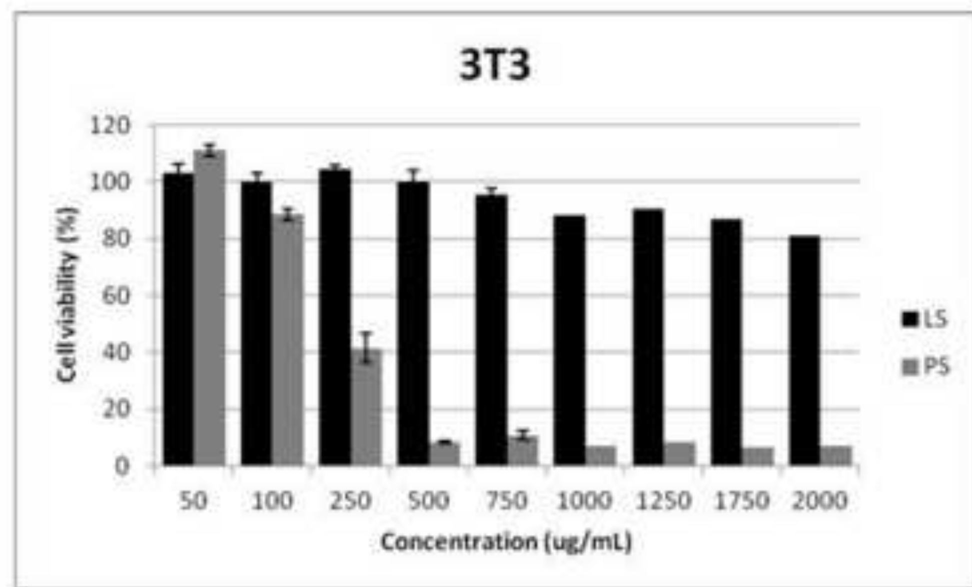

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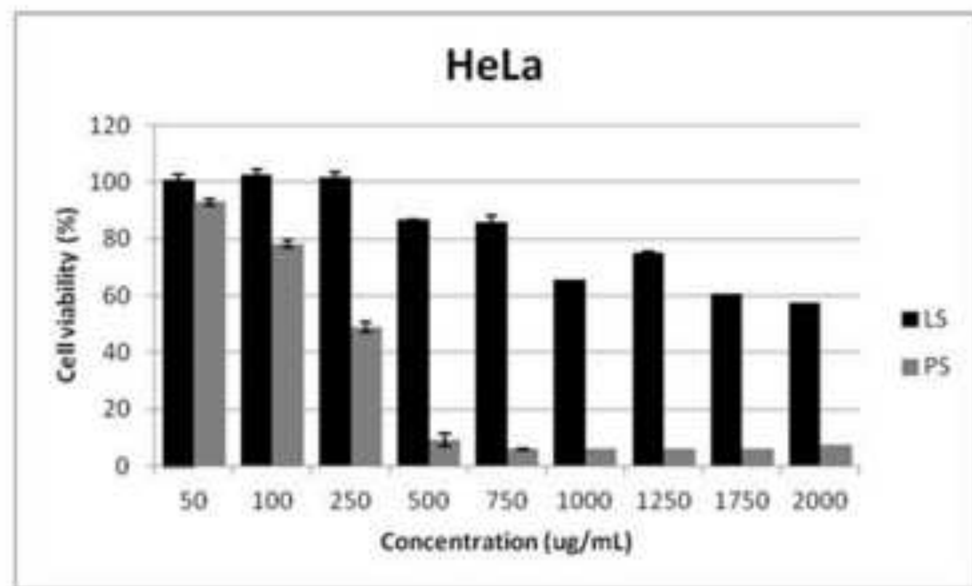

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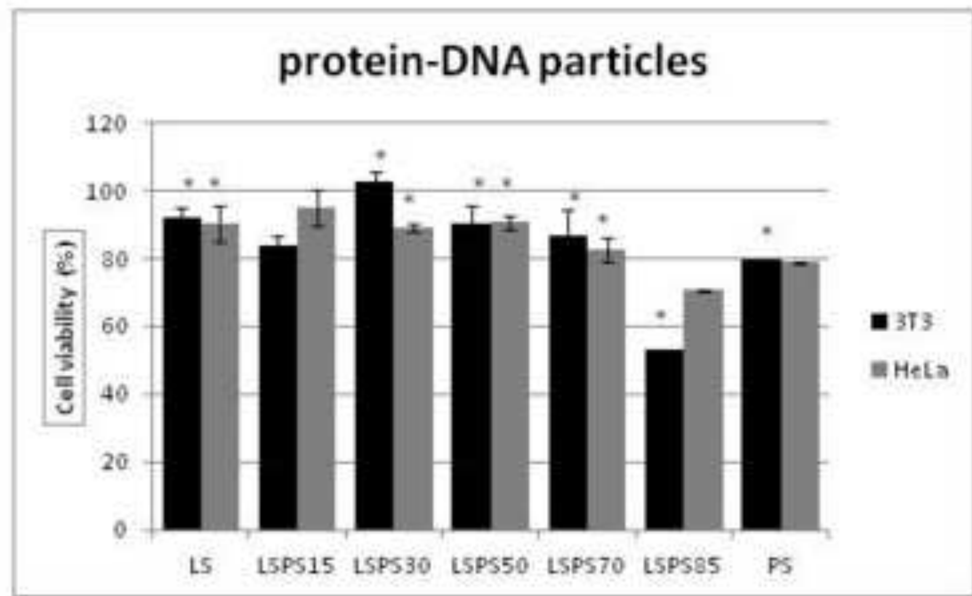

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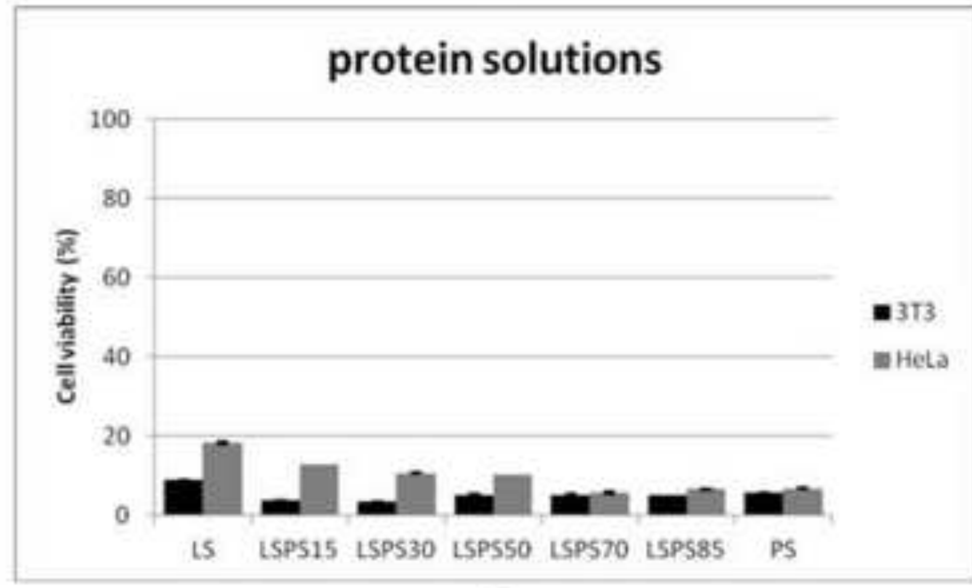

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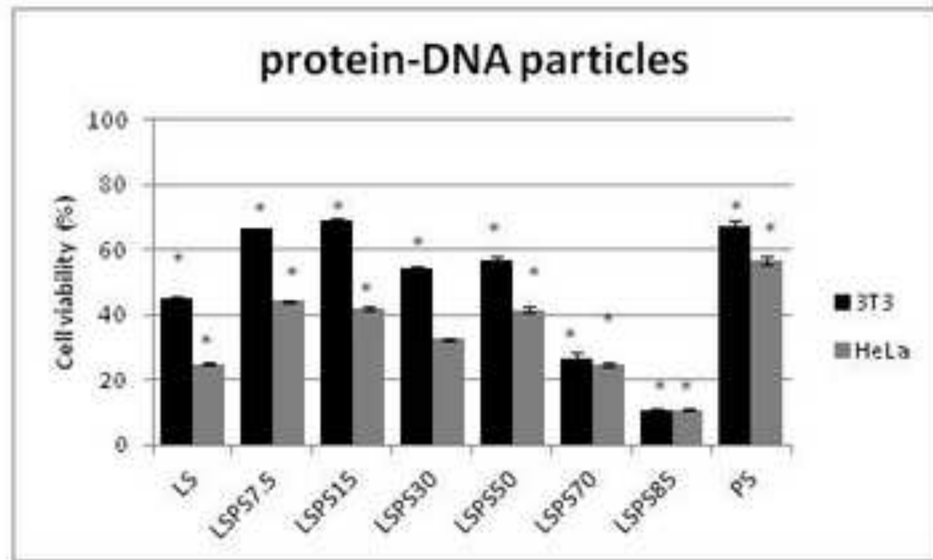

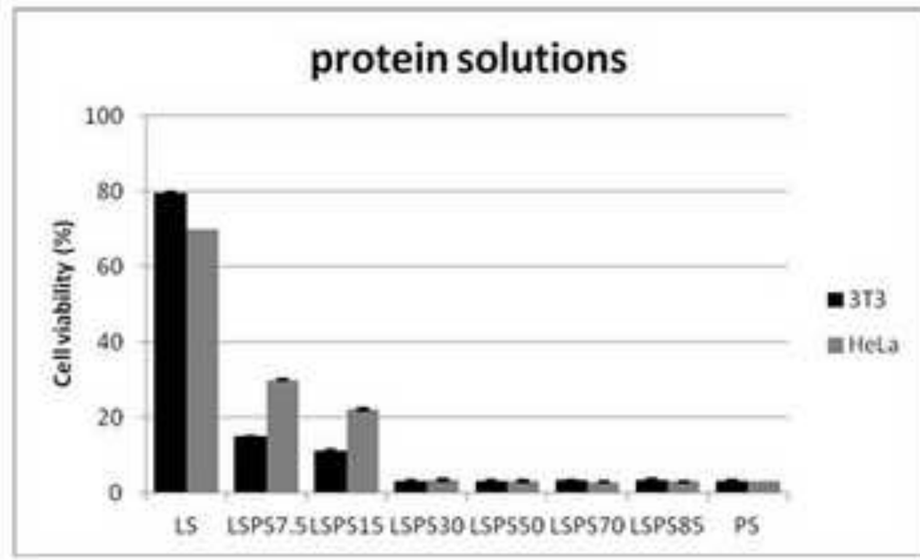

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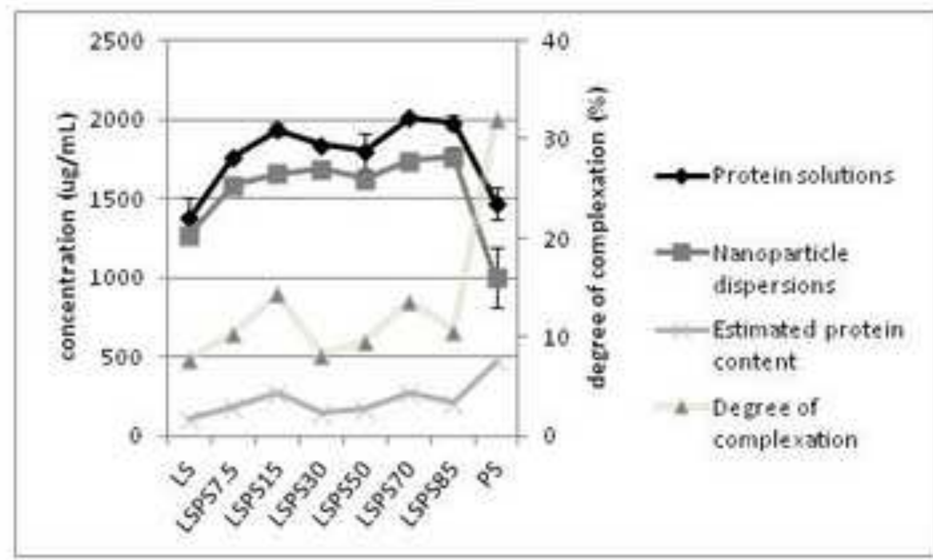

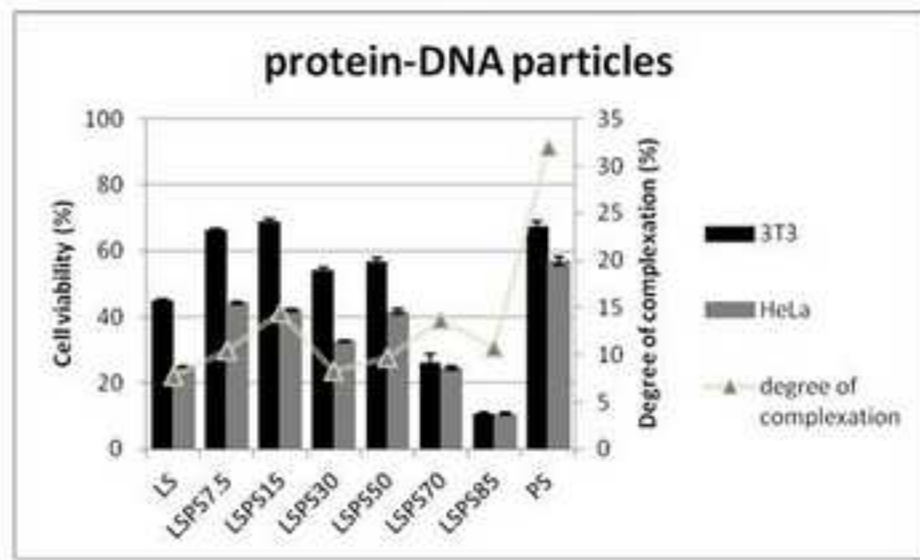

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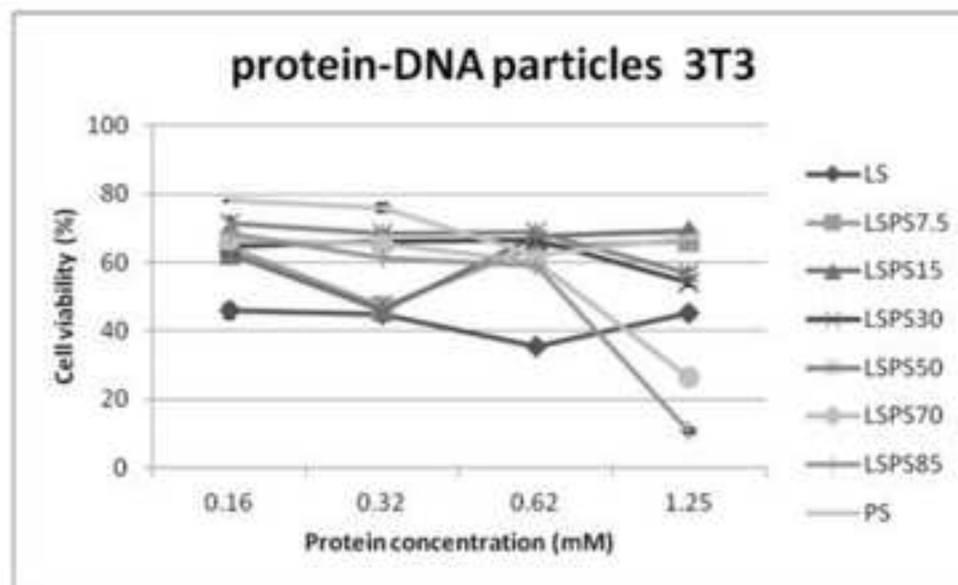
Figure 8



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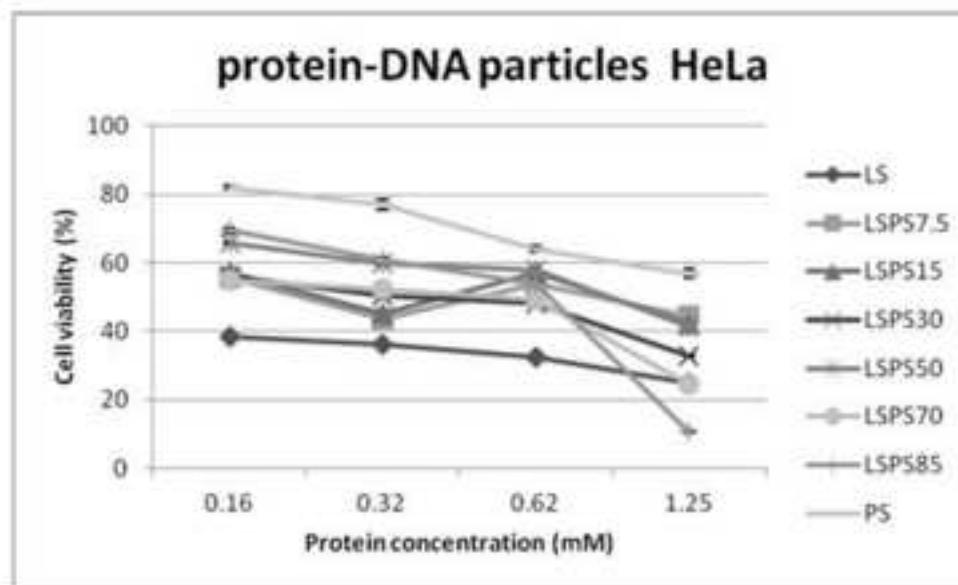

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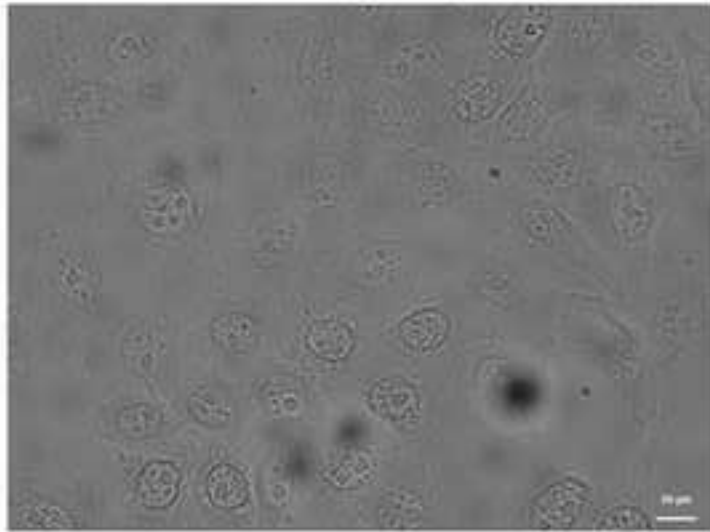

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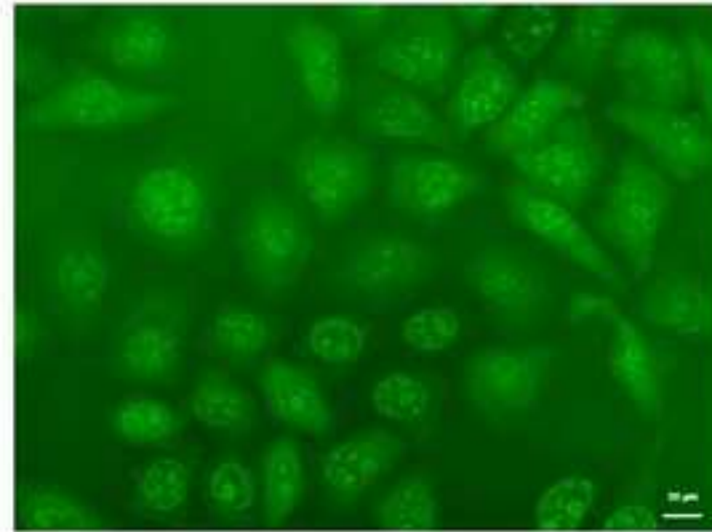

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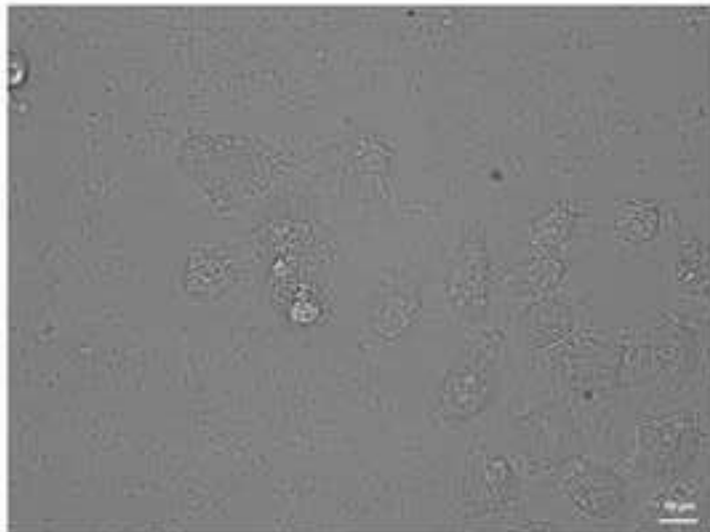

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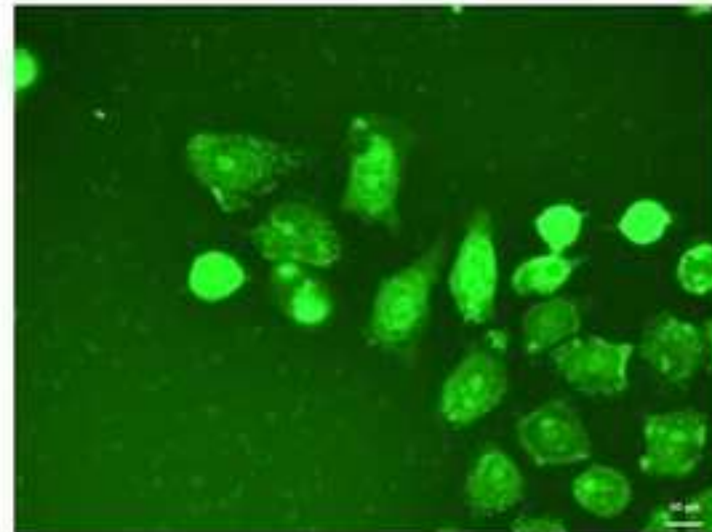

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