

1 **Abstract**

2 The growing wealth of information regarding the influence that physicochemical characteristics play
3 on nanoparticle biocompatibility and safety is allowing improved design and rationale for their
4 development and pre-clinical assessment. Accurate and appropriate measurement of these
5 characteristics accompanied by informed toxicological assessment is a necessity for the
6 development of safe and effective nanomedicines. While particle type, formulation, and mode of
7 administration dictate the individual causes for concern through development, the benefits of
8 nanoformulation for treatment of the diseased state are great. Here we have proposed certain
9 considerations and suggestions which could lead to better informed pre-clinical assessment of
10 nanomaterials for nanomedicine, as well as how this information can and should be extrapolated to
11 the physiological state of the end user.

12 **Key Words**

13 Nanoparticles, Nanotoxicology, Nanomedicine

14

15 **Introduction**

16 The application of nanotechnology in a healthcare setting offers many novel therapeutic strategies
17 that may improve existing therapies and diagnostics. Desirable physicochemical characteristics (PCC)
18 of nanoparticles that can translate to medical benefits include structural and stability related
19 properties to improve bioavailability, biodistribution and reduce clearance [1, 2]. Additionally, there
20 are opportunities for targeted therapies, which may reduce undesirable effects in other cell types,
21 and co-formulation that may alleviate pill burden in diseases such as HIV as well as simplifying
22 dosing strategies by enabling parenteral long-acting depot formulations.

23 While there are obvious advantages to the application of nanotechnology, it is entirely possible that
24 it will not be a case of “one size fits all” and that certain drugs may only be compatible with
25 particular nanoparticles or nanoformulation strategies. Indeed, nanomedicine has attracted recent
26 interest in the fields of precision- and personalised-medicine [3].

27 Size, charge, hydrophobicity and shape are some of the numerous characteristics that can be tuned
28 by the manufacturing process. Modification of these properties can alter the biological interactions
29 of these nanoparticles. For example, uptake of gold nanoparticles by epithelial cells has been shown
30 to be size-dependent where the rate increases with decreasing nanoparticle size [4], and
31 hydrophobic modification of glycol chitosan nanoparticles increased uptake in cancer cells [5].

32 The heterogeneity of nanoparticles being produced by various inventors is a major advantage as it
33 provides many options for the treatment of a broad range of diseases by enabling many strategies
34 for the formulation of therapeutic compounds as well as allowing interactions with many
35 therapeutics. However, the broad spectrum of nanoparticle classes, in addition to their
36 physicochemical characteristics, presents a challenge in determining their biocompatibility. A
37 balance should be found between nanoparticle characteristics that favour the delivery of
38 therapeutic agents while simultaneously not resulting in issues around either toxicity or undesirable
39 interactions with the immune system. Clearly therefore, a rational understanding of how

40 nanoparticle physical properties relate to their biological interactions is required for the efficient
41 development of beneficial materials.

42

43 **Interaction of nanoparticles with components of the immune system**

44 There are many well-described interactions of nanoparticles with cells of the immune system [6].
45 The reasons for these interactions may be linked to specific nanoparticle properties, in particular size
46 and charge [7-9]. Many nanoparticles are within the size range of microorganisms that the immune
47 system has evolved to recognise, with many signatures in common with invading pathogens [10].

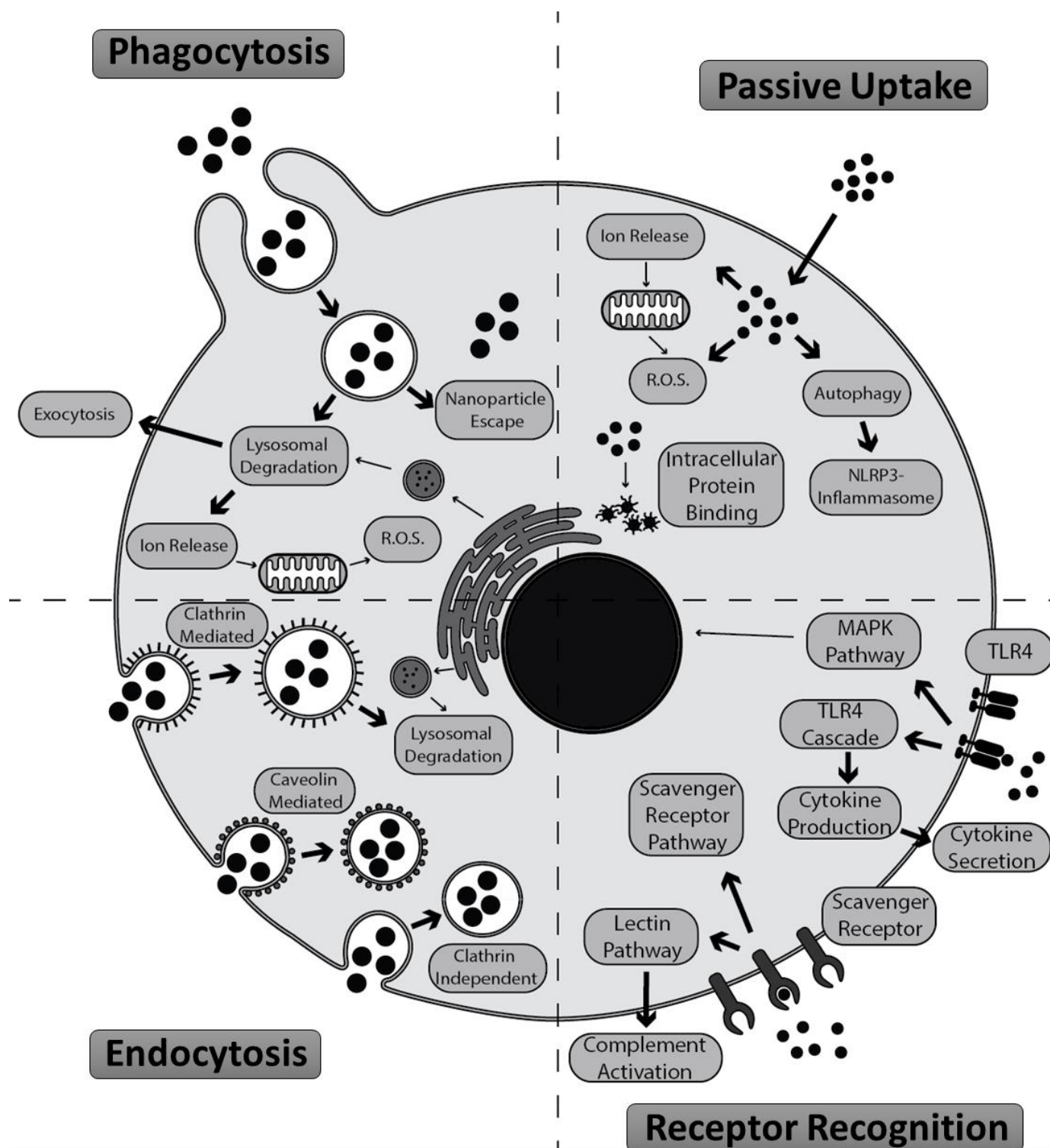
48 The mechanism by which nanoparticles are internalised varies between immune cell types. As
49 demonstrated in **Figure 1** this includes, but is not limited to, phagocytosis, endocytosis, passive
50 uptake, and receptor-interaction based uptake. Phagocytosis (a process performed by macrophages,
51 monocytes, neutrophils, dendritic cells, and mast cells) leads to the capture and internalisation of
52 nanoparticles in phagosomes which in turn undergo lysosomal degradation [11]. While this is an
53 effective tool for removing biological pathogens, nanoparticles are not so simply degraded. The pH
54 environment of the phagolysosome may affect the stability of the nanoparticle leading to the
55 release of metallic ions in the case of metallic nanoparticles [12]. These in turn can disrupt
56 mitochondrial processes and generate reactive oxygen species through Fenton type reactions [12]. A
57 similar effect can be observed in clathrin-mediated [13] and clathrin-independent endocytosis [14]
58 where degradation occurs following lysosomal fusion with the endosome. Caveolin-mediated
59 endosomes bypass lysosomal degradation [15] the mechanism of which is being explored for its
60 potential for intracellular delivery of nanomaterials [16].

61 Nanoparticles which passively enter the cell, or those which escape phagocytic/endocytic vesicles
62 are then able to come in direct contact with intracellular proteins and organelles [17], with the
63 potential to interact in a detrimental manner. Internalised nanoparticles have been shown to
64 interfere with the normal autophagic process [18] and also as a result modulate the NLRP3
65 inflammasome [19].

66 Interaction with certain classes of cell surface receptors leads to the internalisation of nanoparticles,
67 usually displaying certain surface motifs [20] although this is not a necessity as scavenger receptors
68 have been shown to bind polystyrene via the action of macrophage receptor with collagenous
69 structure (MARCO) [21]. Activation of receptor associated pathways as a result of the binding of
70 nanoparticles has been demonstrated where TLR4 signal transduction following the binding of
71 polyethylenimine-coated SPIONs [22].

72 In addition to size and charge, hydrophobicity has also been demonstrated to be an important factor
73 in the recognition of nanoparticles by the immune system [23]. As many intracellular danger-
74 associated molecular patterns (DAMPs) are hydrophobic in nature their release upon cellular
75 damage signals to the immune system to respond to this damage. Hydrophobic nanoparticles have
76 been shown to more likely induce an immune response than those which are less hydrophobic [24].
77 As more classes/types of nanomaterials are created it is entirely possible that additional
78 nanoparticle characteristics will be recognised for their association with biocompatibility, and

79 nanoparticles may be stratified for their interactions with the immune system by class-specific
80 properties.



81
82 **Figure 1 – Routes of entry determine nanoparticle intracellular effects, and extracellular**
83 **consequences.** Internalisation of nanomaterials includes, but is not limited to, endocytosis (including
84 phagocytosis), receptor-binding, and passive uptake. The fate, and associated intracellular effects of
85 these mechanisms include lysosomal degradation, generation of by-products such as metal ions
86 which can induce reactive oxygen species generation in mitochondria, direct interference with

87 *intracellular processes involved in autophagy and the NLRP3-inflammasome, and activation of*
88 *intracellular cascades such as the scavenger receptor pathway, TLR4 cascade, MAPK pathway, and*
89 *the lectin pathway. Extracellular consequences include exocytosis, cytokine secretion, and*
90 *complement activation. Effects displayed here are non-exhaustive, some being ubiquitous and not*
91 *limited to individual modes of entry to the cell. Acronyms used; R.O.S. - reactive oxygen species,*
92 *NLRP3 - NLR family pyrin domain containing 3, TLR4 - Toll-like receptor 4, MAPK - mitogen activated*
93 *protein kinase.*

94

95 Stimulation of the immune system by nanoparticles

96 As foreign substances to the body, nanoparticles may be recognised by the immune system and
97 removed with the possibility to stimulate immune responses by both innate and adaptive
98 mechanisms. Immunogenicity of nanomaterials is largely reliant on their route of administration, as
99 this greatly affects their presentation to the immune system [25].

100 Intravenously administered nanomedicines come directly into contact with plasma proteins which,
101 depending on; particle characteristics, composition and the method of preparation result in protein
102 binding to the nanomaterial surface [26, 27]. While the formation of a “protein corona” is ubiquitous
103 to all nanomaterials when subjected to a biological medium, it has been shown to have important
104 implications for many aspects of nanoparticle-biological interactions *in vivo* [28] such as activating
105 complement [29], and differential cellular uptake dependent on coronal composition [30]. Recent
106 work by Tenzer *et al.* [31] has furthered the understanding of the temporal composition of the
107 nanoparticle corona. While this work was unable to also investigate the “soft corona”, the presence
108 of which further increases the complexity of nanoparticle presentation to the immune system, it has
109 shown that the coronal structure changes as a function of time affecting the material’s
110 pathophysiology.

111 Currently, nanoparticle antigenicity is not well understood. The process of antigenicity involves
112 plasma B cells to generate antibodies against the nanoparticle, or functional groups, such as
113 peptides, attached to the particle surface [32]. Since nanoparticle specific antibodies should only
114 influence the effectiveness of particle-based products, for example by modulating cellular
115 interactions or biodistribution, it is more probable that antibodies that recognise the functional
116 ligands present on the nanoparticle surface may cause similar clinical results as those seen for
117 biotechnology-derived therapeutics [33, 34]. Anti-nanoparticle immunoglobulin formation has been
118 reported. Polyclonal C₆₀-specific antibodies with a subpopulation cross-reacting with the C₇₀
119 fullerene have been demonstrated, as well as monoclonal antibody responses to C₆₀ fullerenes [35,
120 36]. PEGylation (the functionalization of nanoparticles with polyethylene glycol chains) has been
121 used to reduce their immunogenic potential, but the production of anti-PEG antibodies has also
122 been reported [37, 38].

123 Examples of specific nanoparticle properties influencing immune stimulation have been reported.
124 For instance, cationic nanoparticles have a greater potential to induce inflammatory responses than
125 neutral or anionic nanomaterials. An example of this are positively charged 4.5 polyaminoamine
126 (PAMAM) dendrimers do not cause the secretion of cytokines by human leukocytes [39] whereas
127 negatively charged liposomes cause the production of interleukin-2 and interferon gamma [40]. CD4

128 expressing T lymphocytes, known as T helper cells (Th), are a key cell type for the secretion of
129 cytokines. Th cells may be divided into TH1 and TH2, which produce Th1-type or Th2-type cytokines,
130 respectively. Several studies have addressed the influence of nanoparticles on Th1 and Th2
131 responses [41-43]. Th1 cells activate and support cell-mediated immunity, killing virally infected or
132 malignant cells while Th2 cells induce humoral immunity and support antibody production by B cells.
133 Large (>1 μm) industrialized particles induce Th1 responses, whereas smaller (<500 nm) particles are
134 linked with Th2 response [44]. In contrast, engineered nanomaterials including 80 nm and 100 nm
135 nanoemulsions [45, 46], 123 nm self-assembled dendrimers [47], 270 nm poly(lactic-co-glycolic) acid
136 (PLGA) [48], and 500 nm PLGA [49] induce Th1 response. Other engineered particles (e.g. 5 nm
137 generation-5 PAMAM dendrimers) do not demonstrate *in vivo* inflammatory reactions, but enhance
138 immunoglobulin production and weakly induce Th2 cytokine production [50]. The potential
139 contradiction in these findings warrants further investigation to establish whether this is due to
140 nanoparticle characteristics or varying experimental approaches.

141 Macrophages are able to phagocytose nanoparticles, the size of which influences the observed
142 stimulatory effects most likely due to size dependent thresholds on the phagocytic capacity of
143 macrophages [51]. Nanoparticles of the range 200-600nm induce IFN γ , favouring a Th1 type
144 response while 2-8 μm particles induce IL-4 secretion and favour a Th2 type response [52]. From an
145 immunological context, this may be linked to the differential uptake of these nanomaterials as
146 smaller nanoparticles may differentially accumulate in macrophages compared to larger
147 nanoparticles [51, 53].

148 Unwanted immune stimulation is a hurdle for the development of some nanomaterials, but it does
149 also present an opportunity for the formulation of certain therapeutics, in particular, antigens to be
150 utilised in vaccines. The use of nanoparticles as adjuvants has been reported by numerous studies.
151 Poly(methyl methacrylate) (PMMA) nanoparticles have been shown to induce long-lasting antibody
152 titres in HIV-2 whole virus vaccine in mice, and the antibody response was 100-fold higher than that
153 of standard adjuvant [54]. Similarly, the levels of specific antibodies produced in the immunisation of
154 animals with colloidal gold conjugated antigens were higher than that generated by classical
155 adjuvants while the amount of antigen required to achieve this response was an order of magnitude
156 lower than for immunisation with a standard adjuvant [55]. The reasons for this may be due to
157 greater accumulation of the antigen in cells such as dendritic cells allowing greater presentation of
158 the therapeutic antigen to the immune system.

159 Concerning the formulation of vaccines, the generation of inflammation is desirable when
160 nanoparticles are targeted to dendritic cells (DCs). DCs have the ability to induce and modulate the
161 immune response. DCs play a key role in the activation of T cells and as such are a principal target for
162 most vaccines. Utilization of "danger signals" in vaccine design (DC activating non-host signals)
163 combined with specific antigen to induce the desired immune response type is a common approach
164 [56]. As mentioned earlier, nanoparticle size can govern their immunostimulatory profile with
165 plasmacytoid DCs (pDCs) showing preferential uptake of nanoparticles <200nm, resulting in the
166 production of IFN α while phagocytosis by monocytic DCs (mDCs) of 500-1000nm particles induced
167 TNF α [57]. Similarly, Gadolinium containing nanoparticles have been reported to possess antitumour
168 activity resulting from their ability to induce the maturation of immature DCs [39]. Stimulation of
169 DCs by TMC-TPP nanoparticles has been shown to induce differentiation of T cells to inflammatory
170 TH17 [58]. As an alternative proinflammatory pathway to TH1- and TH2-type responses the IL-17

171 mediated cascade offers a further mechanism for enhanced effect as an adjuvant. The opposite
172 effect was observed following DC stimulation by PLGA nanoparticles where not only was TH17
173 differentiation inhibited but also differentiation of naïve CD4⁺ T cells to FoxP3⁺ T cells (Treg cells) was
174 observed. The anti-inflammatory role which Treg cells play in self-antigen tolerance, inhibition of T
175 cell response, cytokine release, as well as NK and CD4⁺ cell activity would not be favourable for a
176 vaccine-based application. Determination of the favourable characteristics of nanoparticles that are
177 correlated with the desired effect is vital to the development of future nanomaterials.

178 The application of knowledge regarding the biodistribution and accumulation of nanomaterials in
179 vivo [59] is highly important when interpreting immunogenicity not only regarding use as adjuvants
180 but for general safety. Passive and active accumulation of nanoparticles in multiple sites increase the
181 concern of off-target toxicity. The relationship between administration route and biodistribution of
182 nanoparticles is intrinsically linked, and to date, there exists no thorough evaluation of route of
183 administration, and how it relates to cytotoxicity following tissue accumulation.

184 Suppression of the immune system by nanoparticles

185 Immunosuppression can be the result of numerous biological effects both directly and indirectly
186 resulting from the systemic presence of nanomaterials. Identification of immunosuppressive effects
187 of nanoparticles is complicated by the fact that these effects may be subtle and not identified until
188 long-term exposure to nanoparticles. Thorough, long-term study is required for the evaluation of
189 immune suppression and careful consideration of the factors involved is required. Unintended
190 immune suppression is an undesirable outcome in areas where patients may already be
191 immunocompromised such as in cancer and HIV infection. Identification of undesirable
192 immunosuppressive properties of engineered nanomaterials may be an important component of
193 their preclinical evaluation. The current knowledge of immunosuppression by nanoparticles has
194 been recently reviewed [60, 61] but some key examples are elaborated in this section.

195 The possible mechanisms by which immunosuppression may occur can be linked to direct anti-
196 inflammatory activity of nanoparticles (silver nanoparticles [62]), nanoparticles with antioxidant
197 activity (cerium oxide nanocrystals [63]), those with anti-cytokine activity (citrate-stabilized gold
198 nanoparticles [64, 65]), inhibitors of cell-mediated immunity (iron oxide nanoparticles [66]), those
199 that interfere with normal antigen response (multi-walled carbon nanotubes [67]), inducers of
200 myelosuppression (doxorubicin bound to polyisobutyl [68]), and those cytotoxic to immune cells
201 (zinc oxide [69]). The range of nanomaterials associated with these outcomes is quite broad, some of
202 which mediate their effects via multiple mechanisms [60].

203 The generation of oxidative stress following accumulation in cells is the primary mode of toxicity for
204 some nanomaterials as demonstrated in **Figure 1**. Generation of reactive oxygen species is linked
205 with activation of the NLRP3 inflammasome [70] which in turn triggers release of proinflammatory
206 cytokines IL-1 β and IL-18 [71], leading to immune stimulation. Certain nanoparticles, including
207 cerium oxide and gold nanoparticles [72, 73], have been found to have antioxidant activity due to
208 their ability to quench free radicals.

209 Nanoparticles such as citrate-stabilised gold have demonstrated anti-cytokine activity by
210 sequestering extracellular IL-1 β [65] thereby inhibiting responses initiated by IL-1 β in certain cell
211 lines. Additionally, interference with TLR9 translocation, via binding of the signalling regulator high-

212 mobility group box-1 (HMGB1) [64], therefore diminishing the effect of TNF α generated by an
213 immune stimulant (CpG-ODN). The binding potential of gold nanoparticles is a commonality that
214 underpins the proposed mechanisms.

215 Fullerenes [74] and carbon nanotubes [67] have been strongly associated with immunosuppression
216 by interfering with the normal response of immune cells to antigens while many dendrimers are
217 being studied to exploit their immunosuppressive qualities [75]. Large amine-and hydroxyl-
218 terminated dendrimers were shown to be able to inhibit inflammation via inhibition of
219 cyclooxygenase (COX1 and COX2) in a concentration-dependent manner [76].

220 API involvement in nano-immunomodulation

221 While inadvertent immunosuppression could result in catastrophic consequences, especially in
222 diseased states with associated immunocompromisation, it may be desirable when utilized in the
223 treatment of inflammatory disorders and autoimmune disease. The clinical potential to improve
224 transplant acceptance by the prevention of allergic responses would be invaluable, and current
225 progress shows great promise by utilizing nanocarriers for the delivery of immunomodulating agents
226 such as rapamycin [77] or donor antigens for the induction of transplant tolerance utilizing
227 vaccine/adjuvant principals [77, 78].

228 Controlled delivery of active pharmaceutical ingredients (APIs) to target sites using nanocarriers is an
229 ongoing challenge. Underpinning this is the need to assess potential and effects of the accumulation
230 of APIs in off-target tissues or immune cells. Polymeric and liposomal carriers are well known to have
231 a higher accumulation in the lymphatic system [79] wherein their potential to interact with
232 lymphocytes in a non-beneficial manner poses cause for concern. Lopinavir, a protease inhibitor
233 used in the treatment of HIV the nanoformulation of which is currently in development [80] has
234 been shown to induce cytokine secretion from various immune cells [81], and Rapamune [82] a
235 nanoformulation of rapamycin used as an immunosuppressant, although possessing antipodal
236 immunological effects are both pertinent examples of APIs whose impacts need to be assessed
237 separately to their carrier system. Following accumulation or degradation of either API or carrier,
238 any associated immunomodulatory effects could become apparent. Immunostimulatory or
239 immunosuppressive properties of the API potentially enhance, or mask those of the carrier system
240 and vice versa. Whether they are by design or unintentional, such effects need to be fully accounted
241 for.

242

243 **Interaction of nanoparticles with components of the blood**

244 Many nanoparticles have been shown to influence a number of haematological components and
245 processes [83]. In their normal homeostatic role platelets facilitate coagulation and are involved in
246 the thrombogenic process to stop bleeding [84]. Platelet activation and thrombus formation have
247 been found to occur in response to nanomaterials in the systemic circulation [85]. Platelet
248 aggregation following the activation of glycoprotein integrin receptor GPIIb/IIIa has been observed
249 for both single walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) in a
250 particle size-dependant manner [85]. Platelet activation has also been strongly associated with
251 GPIIb/IIIa activation by silver ions released from silver nanoparticles [86, 87] and increased

252 intracellular calcium ion concentration resulting from silica nanoparticles [88]. The interaction of
253 charged polystyrene latex nanoparticles has been found to cause physical bridging of platelets in a
254 GPIIb/IIIa independent manner [89].

255 The properties of size, charge, hydrophobicity, and the presence of certain surface groups can
256 determine thrombogenicity of nanoparticles resulting from altering prothrombin times and activated
257 partial thromboplastin times, as well as the mechanism by which coagulation is induced [83]. Anionic
258 polystyrene latex nanoparticles caused platelet aggregation via upregulation of adhesion receptors
259 while their cationic counterparts initiated platelet aggregation following destabilization of cell
260 membrane integrity [90]. Amine-functionalized nanoparticles reduced thrombin production via
261 depletion of factors VII and IX in a size dependent manner [91]. It has been shown that these
262 characteristics hold greater influence over thrombogenicity than does the basic composition of a
263 given material [83]. Cationic, but not neutral or anionic, PAMAM dendrimers cause platelet
264 aggregation [92, 93]. The size-dependence of polystyrene nanoparticles to cause coagulation has
265 been suggested because 220nm but not 24nm particles exhibited this effect [91].

266

267 **Links between immunological and haematological systems**

268 Immunological and haematological systems do not function in isolation and have evolved to work
269 cooperatively to both detect infection and ensure resolution of the response. There are a number of
270 examples of how nanoparticles interact with one system, which in turn activates the other.

271 Leukocyte procoagulant activity

272 Leukocytes play key roles in the regulation of thrombin formation [94] having an influence over
273 inflammation, wound healing and atherosclerosis. Monocytes and neutrophils [95] are recruited by
274 activated platelets at sites of thrombogenesis. This is achieved via recognition of P-selectin on the
275 activated platelet by leukocyte P-selectin glycoprotein ligand (PSGL)-1 resulting in conformation
276 changes in $\beta 2$ integrins [96] leading to potent procoagulant activity. Induction of tissue factor
277 synthesis, the presence of which is necessary for the production of thrombin, leads to thrombus
278 formation [97].

279 Contamination of materials can have a great effect on the pro-coagulant activity of leukocytes. It has
280 been shown that the presence of endotoxin confers leukocytes with considerable procoagulant
281 activity [98]. Contamination of nanomaterials by endotoxin may cause false positives in many
282 immunological assays and it has been demonstrated that cationic PAMAM dendrimers have been
283 shown to enhance the procoagulant activity induced by endotoxin [99, 100].

284 Complement activation

285 The complement system is a vital component of the innate immune system with functions involved
286 in homeostasis, pathogen recognition, and determining the appropriate immune response be it
287 innate or adaptive [101]. Nanoparticles have been shown to activate the complement system
288 following intravenous injection [102]. It is a multicomponent system made up of over 30 membrane-
289 associated and soluble proteins [103]. Complement activation leads to sequential reactions resulting
290 in the formation of C3a and C5a anaphylatoxins which exert multiple inflammatory responses which

291 include the recruitment of phagocytes [103]. Numerous studies have pointed towards complement
292 activation being a contributing factor in the development of hypersensitivity and anaphylaxis as a
293 response to the systemic presence of nanoparticles [6, 104, 105]. Hypersensitivity reactions have
294 been reported for the liposomal formulation Doxil™ [106] there is evidence that this is mediated by
295 complement activation [105]. It has been described that polymeric nanoparticles consisting of PEG-
296 PL (block copolymers of poloxamer and poloxamine) can activate complement exclusively via the
297 lectin pathway [107]. This mechanism is normally reserved for the recognition of repeating and
298 charged motifs of certain polysaccharides [108].

299 Platelet activation and immune stimulation

300 The link between platelet activation and immune stimulation is multifactorial and double-edged.
301 While thrombogenesis can influence immune stimulation, along with various thrombogenic factors
302 being able to inhibit or augment immune responses, the opposite is also true where immune
303 stimulation increases thrombogenic potential. Proinflammatory cytokines and endotoxin induce
304 tissue factor production on leukocytes which in turn initiates extrinsic coagulation via thrombin (FIIa)
305 generation [109]. Complement activation leads to enrichment of plasma membrane surfaces with
306 negatively charged phospholipids which have been shown to amplify coagulation [110].

307 Thrombogenic function is just one of the numerous activities which platelets can play within
308 homeostasis. The involvement of platelets within immune stimulation has gained recognition in
309 recent years [111, 112]. Platelets carry numerous receptors including TLRs and express
310 immunomodulatory molecules and cytokines [113]. An example of how nanoparticles may cause
311 immune stimulation via platelets has been demonstrated previously with multi-walled nanotubes
312 (MWNT). MWNT were shown to induce the release of platelet membrane microparticles capable of
313 stimulating other immune cells [114]. Further studies are warranted on the interaction of platelets
314 and immune cells with respect to nanoparticle effects on both cell types.

315 Haemolytic potential

316 The mechanisms of nanoparticle-mediated haemolysis are not fully understood. Haemolysis is the
317 result of damage to red blood cells and may be used as a measure of cell viability in response to
318 contact with materials in addition to possibly leading to anaemia [115]. Many studies currently exist
319 which describe the haemolytic potential of various nanomaterials but only some suggestions exist
320 concerning their mode of action [104] primarily membrane disruption via interactions with red blood
321 cell membrane phosphatidylcholine [116, 117]. Charge has been shown to strongly influence
322 whether nanoparticles cause haemolysis. This process has been related to the disruption of cell
323 membranes via pore formation following the integration of charged nanoparticles into existing
324 membrane defects [118]. The potential for nanoparticles to become ionised [119], surface groups
325 [116, 117], and cationic charge seem to be parameters likely to have an effect. Materials which
326 exhibit this trend include silica nanoparticles [120, 121] as well as numerous others via the presence
327 of unprotected amines on the nanoparticle surface such as PAMAM [122], carbosilane [123],
328 polypropylene imine [124], and polylysine [125] dendrimers, which have been associated with
329 erythrocyte damage in a dose dependent manner. The haemolytic potential of silver nanoparticles
330 has been well described in numerous sources [86, 119, 126]. It has been demonstrated that with
331 increasing hydrophilicity the haemolytic potential increases [127]. The presence of a protein corona
332 has been shown to have a protective effect, and the haemolytic potential of gold nanoparticles

333 featuring both hydrophobic and hydrophilic surface functionalization was reduced [127]. This effect
334 has also been described by Tenzer *et al.* wherein the presence of protein corona on silica
335 nanoparticles negated their haemolytic activity as well as a reduced level of thrombocyte activation
336 compared to pristine nanoparticles [31].

337

338 **Challenges in assessing the biocompatibility of novel, engineered, nanoparticles**

339 Contamination

340 The potential for nanomaterial contamination is intrinsically linked to the associated manufacturing
341 process. Bacterial endotoxin is a contaminant which elicits a strong immune response upon
342 exposure [128]. Endotoxin is a component of Gram-negative bacterial cell walls and can contaminate
343 nanomaterials during the manufacturing process or in handling. It has been shown that endotoxin
344 can exacerbate inflammatory responses to nanoparticles [129-132]. As a result of the potent
345 proinflammatory activity the presence of endotoxin in nanomedicines whose administration to
346 individuals in an already diseased state leads to the question of how this, in combination with
347 potential nanoparticle associated immunomodulation, may affect an already compromised immune
348 system.

349 The formulation of nanomedicines can represent complicated, multistep processes often involving
350 the use of volatile chemicals and reagents. These volatile agents must be removed to prevent
351 toxicity being generated by carry-over from contaminants within the formulation process [133]. The
352 cytotoxic analysis of a preparation of gold nanorods both pre- and post-purification has
353 demonstrated the stark contrast which can be the result of residual manufacturing components
354 [134]. This observation has also been described by some sources where the toxicological potential of
355 carbon nanotubes has been assessed [135, 136]. The production of carbon nanotubes requires
356 catalysis by transition metals [137]. Most frequently these are iron, nickel, and copper. As free ions,
357 these metals have been shown to induce oxidative stress via the production of reactive oxygen
358 species (**Figure 1**) [138, 139]. Chemical contamination of this type has been detected in commercially
359 available preparations of carbon nanotubes where, following purification, the material was no
360 longer deemed toxic [140].

361 Nanoparticle interference with assays

362 A number of *in vitro* assays have been adopted for use with nanomaterials [141]. Their translation to
363 use in nanotoxicology is mainly due to their track record of versatility, simplicity, and reproducibility.
364 As has become apparent in recent years; the appropriateness to apply these methodologies with
365 little consideration to how novel materials may lead to spurious assay outcomes [142]. Determining
366 the appropriateness of assays for this end is complicated by the intrinsic complexity of nanoparticles.
367 As such, suitable inhibition/enhancement controls (IEC) should be included in this analysis when
368 possible.

369 Adsorption of protein to the surface of nanoparticles reduces the concentration of free protein
370 available for quantification. The polarity of nanoparticles can enhance or reduce their potential for
371 binding proteins from a matrix. This is particularly evident by the reduction in measurable IL-8 due to
372 adsorption to a titanium dioxide preparation [142]. Similarly, TLR9 and IL-1 β binding to citrate-

373 stabilized gold nanoparticles has been documented [64, 65]. The ability of nanoparticles to interact
374 with, and inactivate enzymes is a consideration which reaches beyond the potential *in vitro* and *in*
375 *vivo* effects. Numerous methods for testing the toxicity of nanomaterials rely on enzymatic function.
376 The potential for interaction dictates that further considerations be made so as not to generate data
377 which may not be representative of the material but merely an artefact of experimental interference
378 [143]. Few assays have been implicated with this form of interference to date. One that has been
379 brought to light is the LDH assay. Inactivation of lactate dehydrogenase as a result of adsorption to
380 nanoparticle surfaces has been presented as a mechanism by which the LDH assay can produce
381 results which are not an accurate representation of nanoparticle action [142, 143].

382 Studying the haemotoxic effects of nanomaterials lends the opportunity for a number of
383 methodological issues relating to the basic properties of nanoparticles under investigation. The
384 turbidity of nanoparticle preparations is known to interfere with platelet aggregometry, the principal
385 of which relies on the optical assessment of the decrease in turbidity due to platelet aggregation. A
386 potential solution for this is to utilize alternative measurement methods such as flow cytometry.
387 Systems utilising magnets, such as those used for measuring platelet activation, have the potential
388 to be incompatible with magnetic nanoparticles. When subjected to the magnetic field a region of
389 higher concentration may establish, the effect of which may skew any observations and not be
390 representative of a uniform distribution.

391 Proliferation is commonly evaluated using the MTT assay, but there are numerous mechanisms by
392 which this can be incompatible with nanomaterials. A potential issue with the use of this assay is
393 that it relies on the metabolic conversion of the MTT compound. Materials which promote/alter
394 mitochondrial biogenesis cause artificially high signal which could be mistaken as pro-proliferative
395 [144]. Differences in rates of tetrazolium production is reflective of the metabolic state of the cells
396 [145, 146]. It is known that activated lymphocytes are more metabolically active than non-activated,
397 which may reflect altered metabolism rather than proliferation [147]. Nanoparticles affecting
398 metabolism and proliferation would be difficult to discern so the use of further methods such as
399 [³H]thymidine incorporation and CFSE should be utilised. Quantification of cytokines as a marker of
400 proliferation can also be problematic as the reduction may be the result of cell death.

401 The issues described here hold equal validity not only for toxicity assays but for immunotoxicity as
402 the reagents employ similar strategies for generation of a measurable result i.e. absorbance,
403 fluorescence. As such, the potential for nanoparticle-based assay interference must be considered
404 throughout assay development and data interpretation.

405 Nanoparticle physicochemical characteristics in biological matrices

406 In order to determine structure-activity relationships and define meaningful trends, it is necessary to
407 accurately measure physicochemical characteristics. The application of nanomaterials under
408 biological conditions, both *in vitro* and *in vivo*, require in-depth knowledge of their physicochemical
409 properties in relevant matrices. Due to the increasing complexity of biological matrices, it is not
410 sufficient to assume that characteristics determined under minimal conditions (i.e. under vacuum, or
411 in water) are still valid in the rational design and development for given purposes. The size, charge,
412 surface chemistry, stability, and a host of other properties can be directly and dramatically altered
413 by the medium in which the nanoparticles are suspended, all of which may affect how the materials
414 interact with biological processes [148, 149].

415 Not only is it important to produce accurate and appropriate determinations of the physicochemical
416 characteristics of nanomaterials, but it must be appreciated that the production of such materials is
417 often a complex multistep process. Changes in particle size and/or charge can affect particle
418 biodistribution, immunological impact and broader aspects of safety for nanoparticles made of the
419 same material [93, 100]. While polydispersity within and between preparations must be expected,
420 this batch-to-batch variability must be strictly monitored and accounted for to minimize
421 downstream issues.

422 The issue of determining biologically meaningful *in vitro* assays which can inform downstream *in vivo*
423 studies is further complicated by the choice of appropriate cellular models and endpoints. A recent
424 review by Dobrovolskaia [150] has examined these considerations in detail, as such will not be
425 repeated here. Linked with this are need to choose relevant and efficacious controls as well as
426 determine any interaction between the nanomaterial and assay itself. To exemplify this issue, it was
427 earlier mentioned that numerous cytotoxicity assays are prone to nanoparticle-related interference.
428 Without detailing the choice of cell line or endpoint the choice of controls and assay interaction
429 potential shall be discussed. The cytotoxic compound of choice must be sufficiently potent within
430 the given cell line to generate toxicity but would ideally have a mode of action similar to that which
431 would be expected from a nanomaterial. While this is desirable, tetrazolium salts such as MTS/MTT
432 which detect the REDOX potential of cells would not be necessarily compatible with ROS generators
433 such as dicumarol which can lead to overestimation of cellular viability and proliferation [151].
434 Similarly, compounds which affect cell membrane integrity should be used with care in the LDH
435 assay, especially when comparing results of different cytotoxicity assays. Cell-free preparations of
436 assays can be considered vital as a means to not only generate a baseline but also to observe any
437 concentration dependent interactions that may occur. This can be invaluable in fluorogenic assays
438 such as DCF where a threshold for interference may exist [152]. As mentioned earlier, the inclusion
439 of inhibition/enhancement controls can assist in determining whether observations are a result of
440 cellular interactions with nanomaterials or solely due to the presence of the nanomaterial. This is
441 becoming routine in limulus amoebocyte lysate (LAL)-based assays for measuring endotoxin in which
442 a nanomaterial sample is spiked with a known amount of endotoxin and assessed for enhanced or
443 diminished recovery [153]. The underlying principal is translatable to a host of assays in which
444 inducers or inhibitors of the desired effect can be introduced in addition to nanomaterials. Although
445 logical, these considerations are widely overlooked potentially resulting in misleading conclusions
446 being drawn.

447

448 **Considerations for specific patient populations**

449 Research efforts examining the biocompatibility of nanomaterials primarily use blood, as well as
450 immune cells, from healthy volunteers to assess potential interactions. However, the intended
451 populations often have differential immunological profiles compared to healthy volunteers. It is,
452 therefore, vital that these aspects be considered when testing novel engineered nanomaterials.

453 The broad concepts of immunological frailty and how they relate to potential interactions with
454 nanomaterials has been described [154] and highlights the relative lack of experimental evidence in
455 such populations compared to investigations in healthy volunteer cells and tissues. There is evidence
456 to suggest that the genetic background of the test organism can influence the outcome of

457 biocompatibility testing. Gustafsson *et al.* [155], showed that the response to titanium dioxide
458 nanoparticles in rats was strain-specific, indicating that genetics plays a role in the response to
459 nanomaterials. Existing data on the effects of nanoparticles in animal models reflecting
460 immunological frailty, dysregulated immunity and immune-compromised states show that
461 nanoparticles can have greater, or an additive, toxicological effect to that resulting from the
462 diseased state [156]. However, how closely animal models can reflect the situation in humans with
463 respect to disease states is an ongoing issue surrounding many fields of research, and it seems likely
464 that obtaining *ex vivo* samples from patients with specific conditions may complement other pre-
465 clinical evaluations, prior to phase I trials.

466 As one would expect, potential side effects and immune interactions by nanomaterials may be
467 further influenced by dysregulation of the immune system as a result of the diseased state. HIV is a
468 pertinent example of this, wherein the disease is underpinned by complex multifactorial
469 immunomodulation, and treatment paradigms are currently being investigated for improvement via
470 the application of nanoformulation [157].

471 There exist several parallels between the immunological effects of nanomaterials and those of the
472 diseased state. These effects include some generated by chronic inflammation such as rheumatoid
473 arthritis, cancer, and even hepatitis and HIV.

474 As mentioned previously, the activation of TH17 type response by TMC-TPP nanoparticles leads to
475 the generation of IL-17 [58]. The generation of this particular proinflammatory factor is of interest in
476 the pathogenesis of rheumatoid arthritis, as its production in the synovial tissue has been shown to
477 promote destructive collagen arthritis in an IL-1 independent manner in murine models [158], and
478 act synergistically with IL-1 and TNF α [159].

479 The pathogenesis of cancer is intrinsically linked to a multitude of cytokines generated by the innate
480 and adaptive immune systems including IL-1, IL-6, IL-12, IFN γ , TNF α [160] all of which have been
481 shown to be associated with the interactions of various nanoparticles including silver (IL-1) [161],
482 MWCNT (IL-6) [162], and zinc oxide (IL-12, IFN γ , TNF α) [69]. As a platform for immunotherapy
483 nanoparticles are being studied due to their known induction of various immunostimulatory
484 cytokines which are proposed to exacerbate, and illicit, a greater immune response against
485 cancerous cells.

486 Mechanisms proposed to result in apoptosis in HCV and HIV-infected cells include loss of cell
487 membrane integrity, mitochondrial dysfunction and generation of ROS [163]. Silica [164] and
488 titanium dioxide [165] nanoparticles have been shown to alter cell membrane integrity in a charge-
489 and concentration-dependent manner. Oxidative stress and the generation of reactive oxygen
490 species is directly relatable to mitochondrial dysfunction (**Figure 1**) [166]. A large number of
491 nanomaterials have implicated with having a similar effect [167]. HIV has been shown to interfere
492 with the autophagic process via inhibition in dendritic cells, and induction in macrophages [168],
493 while HCV has shown to increase levels of autophagy in infected cells [169]. Inhibition [170] or
494 induction [171] of autophagy by nanomaterials (**Figure 1**) is also a commonality to the actions of HIV
495 and HCV. Therefore, it seems likely that certain material compositions should not be progressed for
496 certain applications.

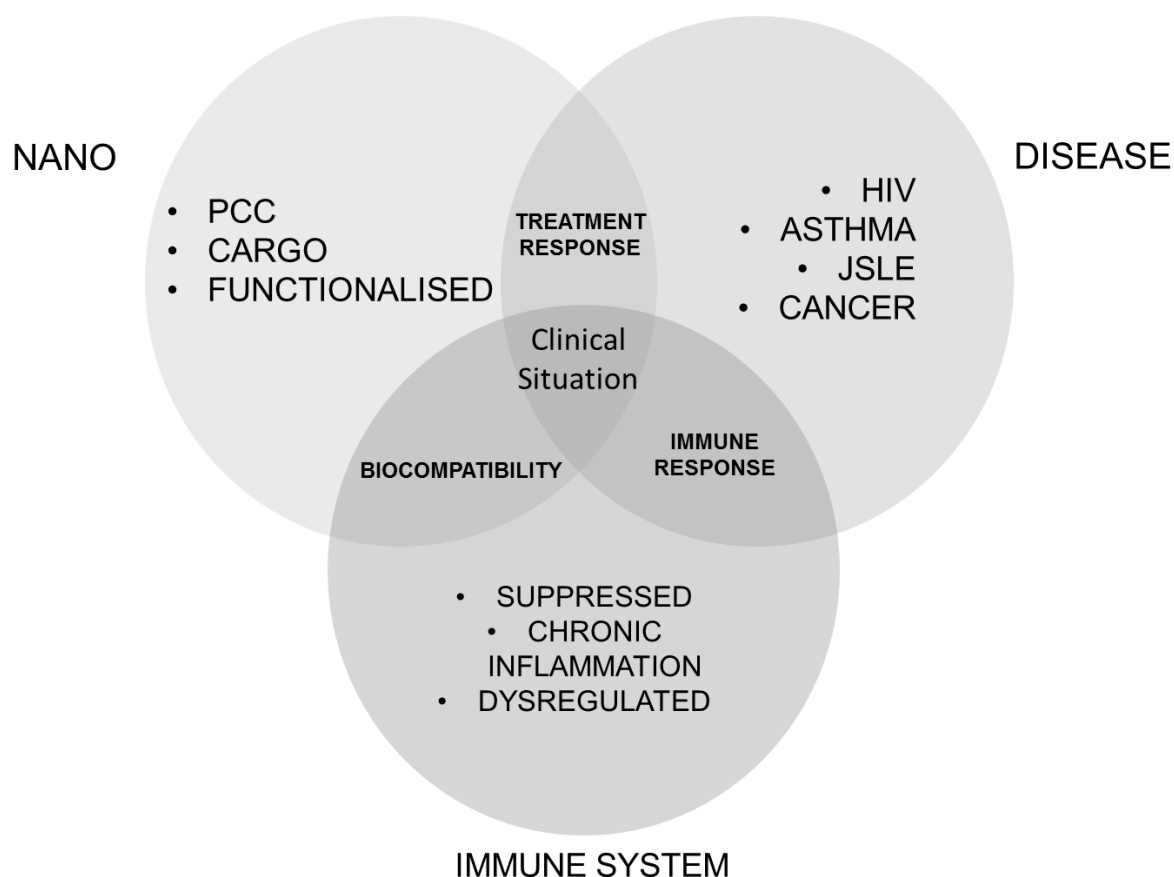
497 Immunocompromised individuals can be defined as having a substantially weakened immune
498 system, and this was originally thought to be the case in HIV infection. However, it is now known
499 that the situation is not clear cut since a patients' immunological profile varies with the type of viral
500 populations infecting them and their response to antiretroviral therapy [172]. Infection with HIV
501 leads to a decline in CD4+ T cells, but treatment with antiretrovirals may produce resurgence in the
502 number of these cells. However, it has been shown that although the number of CD4 T cells
503 increases their functional capacity is diminished in chronic infection. This has been demonstrated by
504 the increased expression of the receptor programmed death 1 (PD-1), a negative regulator of
505 activated T-cells [173]. Cells expressing high levels of PD-1 were shown to be functionally exhausted
506 compared to uninfected cells suggesting HIV+ patients are immunocompromised [174]. However,
507 the reasons for this exhaustion of the immune system are unclear, and several hypotheses have
508 been proposed [175]. An interesting hypothesis for the ongoing inflammation seen in HIV, which
509 may be linked to T cell exhaustion, is the discovery that HIV itself can induce an inflammatory form
510 of programmed cell death termed pyroptosis. Dotish *et al.* showed that HIV can directly induce
511 pyroptosis in CD4 T cells via inflammasome activation and that this process could be blocked by
512 inhibiting caspase-1 [176]. Interestingly, nanoparticles have been shown to interact with
513 inflammasomes, NLRP3 in particular (**Figure 1**) [177] and carbon nanoparticles have been shown to
514 induce pyroptosis [178]. This is an important consideration for the application of nanoparticles
515 either in the treatment of HIV infection or when nanoparticles may be applied in HIV+ patients for
516 concomitant health issues, e.g. raised cholesterol or infections. As a condition where chronic dosing
517 is a reality which cannot be overlooked, the long term effects of any nanoformulation must be
518 considered and is something we are investigating with interest.

519 Effects such as these may be tolerable in a healthy model but be potentially incompatible with the
520 diseased state. It is also possible for the opposite to be true, where the observable effect is
521 unacceptable under healthy conditions, whereas its effect on the diseased state may not be as
522 pronounced and within a range where the potential benefits outweigh the negative outcomes. As is
523 demonstrated in **Figure 2** the primary considerations of the nanomaterial itself, the immune system
524 to which it will be introduced, and the disease on which it will act are not mutually exclusive. The
525 intersections of biocompatibility and treatment response are those which weigh heavily in the
526 development of nanomedicines. Often overlooked is the immune response relating the disease to
527 the immune state, and also how the nanomaterial has influence over these. To be able to create a
528 truly appropriate model for the design of nanomedicines, a holistic approach such as this must be
529 adopted.

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534 **Figure 2 - Key challenges in compatibility of nanoparticles as nanomedicines.** Considerations
 535 involved in the design, analysis, and application of nanomaterial for the treatment of disease linking
 536 the material specific, immune state, and particular disease. A more holistic approach incorporating
 537 investigation of immunological status and genetic variability in genes encoding immune signalling
 538 proteins will allow a more holistic approach to the biocompatibility testing of novel engineered
 539 nanomaterials. Acronyms used; PCC – physicochemical characteristics, HIV – human
 540 immunodeficiency virus, JSLE – juvenile systemic lupus erythematosus.

541

542 **US and EU efforts to promote the harmonization of nanoparticle testing**

543 To truly determine relationships between nanoparticle characteristics, the necessity to apply a more
 544 standardised approach to assays has become apparent in order to correctly assess how
 545 nanoparticles interact with biological systems. Many researchers involved in the development of
 546 nanomaterials use well-defined assays to assess biocompatibility e.g. investigation of cytotoxicity by
 547 using MTT assays. However, there are reports of contradictory test results from cell-based assays
 548 [179, 180]. Unexpected variability can arise in such assays by differences in media composition,
 549 passage time of cell lines and the source of the serum used in routine cell culture media. The
 550 National Cancer Institute’s Nanotechnology Characterisation Laboratory (NCI-NCL)
 551 (<http://ncl.cancer.gov/>) has been at the forefront of promoting harmonisation of assays to
 552 determine nanoparticle interactions with biological systems and offers standardised methodologies
 553 for its assessment. Given the increasing development of nanomaterials across Europe, a need has
 554 been identified to begin to regulate the preclinical evaluation of novel engineered nanomaterials as

555 well as provide a platform for the translation of these materials into clinical studies. The recently
556 established European Nanomedicine Characterization Laboratory (EU-NCL) (<http://www.euncl.eu/>)
557 shares the same ethos as the NCI-NCL in the provision of a standardised characterisation of
558 nanomedicines to aid in their translation to the clinic and facilitate nanomedicine development.
559 Currently, researchers and developers in Europe have to gather preclinical data from a multitude of
560 non-integrated providers which may result in interlaboratory variability and, therefore, conflicting
561 results. A major ambition of the EU-NCL is to tackle that obstacle by providing an open-access EU-
562 wide characterisation infrastructure and maintain Europe as internationally competitive in
563 nanomedicine development. EU-NCL offers a unique integrated solution ensuring access to high-
564 quality data, experience, and facilities throughout Europe for a large range of medical applications.
565 EU-NCL is a multi-centre infrastructure which is intended to overcome current fragmentation and to
566 improve quality and efficiency of translation by drawing on expertise across Europe. The
567 involvement of multiple analytical centres guarantees direct access to different domains in the
568 nanomedicine communities and other stakeholders while maintaining the bandwidth to engage with
569 Europe's most promising candidates. It is envisaged that using this integrated approach, EU-NCL will
570 also be able to determine critical nanoparticle characteristics that relate to biological effects,
571 without compromising confidentiality with developers. As such, this will enable researchers to
572 access anonymised information to inform future rational design of nanomaterials.

573

574 **Conclusions and future perspectives**

575 The development, and implementation, of nanomaterials for a variety of clinical applications is
576 increasing as their utility in improving healthcare is demonstrated. However, consideration must be
577 given to appropriate pre-clinical testing to fully translate these materials into clinical use.

578 Numerous conclusions can be drawn from existing research, among which are perspectives on how
579 pre-clinical testing can be improved from its current state. As mentioned here thorough
580 physicochemical characterisation in biologically relevant matrices is vital, similarly assessing the
581 contamination state of products. These need to be supported by biologically meaningful *in vitro*
582 assays which can inform further *in vivo* studies. Linked with this are need to choose relevant and
583 efficacious controls as well as determine any interaction between the nanomaterial and assay itself.
584 Greater insight into the effect of nanoparticles on the diseased state would benefit from testing in
585 relevant patient samples. Finally, the nanomaterials should be considered in the final format for
586 which they have been developed. Not only will this aid in determining if the nanoparticle is fit for
587 purpose, but also how its application may affect patient populations in terms of nanomedicine.

588 It is hoped that with greater integration and cooperation of various research efforts the
589 development of nanomedicines will gain speed to bring forward these advances in patient care.

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592

593 **Executive Summary**

594 **Introduction**

- 595 • Nanoformulation provides a platform which allows improvement over existing therapeutic
596 and diagnostic tools.
- 597 • Physicochemical characteristics of nanomaterials can be tuned during the manufacturing
598 process as a means to enhance/reduce physiological effects.
- 599 • Challenges in the characterisation of nanoparticles relating to biocompatibility relate to
600 many factors including different manufacturing processes, and the immune state of the end
601 user.

602 **Interaction of nanoparticles with components of the immune system**

- 603 • Various mechanisms, the biological purposes of which under normal circumstances are
604 homeostatic or relating to clearance of pathogens, are known to be implicated following the
605 introduction of nanomaterials to biological systems.
- 606 • While mechanisms of internalisation of nanomaterials differ as a result of cell type, as well
607 as physiochemical characteristics i.e. size and charge, downstream effects such as the
608 generation of reactive oxygen species etc. can be ubiquitous.
- 609 • Factors such as protein corona formation, although not well understood, are shown to
610 modulate biological interactions, uptake, and overall pathophysiology.
- 611 • Inflammatory stimulation of the immune system, antibody production against certain
612 materials are known examples of interactions which may be detrimental to the host.
- 613 • Immunosuppressive properties of certain nanomaterials associated with certain
614 nanomaterials could potentially exacerbate the pathophysiology of immunocompromised
615 individuals.
- 616 • Complexity in these considerations is increased by the presence of active pharmaceutical
617 ingredients.

618 **Interaction of nanoparticles with components of the blood**

- 619 • Interactions of nanoparticles with haematological components can lead to modulation of
620 thrombogenic potential.
- 621 • The complexity of these interactions is a function of the physicochemical characteristics of
622 the nanomaterial as well as the multifactorial nature of the process of thrombogenesis.

623 **Links between immunological and haematological systems**

- 624 • The cooperation of immunological and haematological systems add complexity to the
625 evaluation of nanomaterial biocompatibility.
- 626 • Leukocyte procoagulant activity is shown as an example where contamination of
627 nanomaterial preparations can strongly generate a false positive.
- 628 • Complement and platelet activation are complex cascades both of which have been shown
629 to be affected by the presence of various nanoparticles.
- 630 • Disruption of membrane integrity leading to haemolysis has been associated with a number
631 of nanomaterials. The presence of a protein corona modulates this activity.

632 **Challenges in assessing the biocompatibility of novel, engineered, nanoparticles**

- 633 • The contamination state of tested materials, both biological and chemical, can skew data by
634 the generation of false positives.
- 635 • The lack of nanoparticle-tailored assays necessitates the use of standard immunological
636 assays, many of which succumb to interference by intrinsic properties of nanomaterials
637 which can lead to spurious results.
- 638 • The necessity to utilize complementary assessment methodologies which focus on particular
639 aspects via differential means has been highlighted.
- 640 • Physicochemical characterisation in biologically relevant matrices has been highlighted as
641 providing a more relevant representation of the material coming in contact with cells.
- 642 • Suggestions have been provided relating to assay combinations and positive control choices.

643 **Considerations for specific patient populations**

- 644 • The immunological state of the intended recipient is of primary importance when
645 considering the application of nanoparticles for nanomedicine.
- 646 • The immunological effects of nanoparticles have the potential to exacerbate those
647 generated by the diseased state.
- 648 • Hallmarks of chronic inflammatory conditions display commonality with those generated by
649 nanomaterials. As such caution must be taken in their use under such conditions.
- 650 • Assessment of nanomaterial safety is normally performed in healthy models and while
651 convenient, does not provide the necessary conditions present in the diseased state.

652 **US and EU efforts to promote the harmonization of nanoparticle testing**

- 653 • International standardisation efforts for nanoparticle characterisation which can aid
654 preclinical evaluation of nanomedicines by addressing the aforementioned challenges in
655 nanomaterial testing

656 **Conclusions and future perspectives**

- 657 • The need for thorough and biologically relevant preclinical testing is reiterated.
- 658 • Consideration of the diseased state in these assessments is of high importance.

659

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