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## DePEGylation strategies to increase cancer nanomedicine efficacy

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### Citation

Kong, L., Campbell, F., & Kros, A. (2018). DePEGylation strategies to increase cancer nanomedicine efficacy. *Nanoscale Horizons*, 4(2), 378-387. doi:10.1039/C8NH00417J

Version: Not Applicable (or Unknown)

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**Note:** To cite this publication please use the final published version (if applicable).

1   **DePEGylation strategies to increase cancer nanomedicine efficacy**

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10   **Abstract:** To maximize drug targeting to solid tumors, cancer nanomedicines with prolonged circulation  
11   times are required. To this end, poly(ethylene glycol) (PEG) has been widely used as a steric shield of  
12   nanomedicine surfaces to minimize serum protein absorption (opsonisation) and subsequent recognition  
13   and clearance by cells of the mononuclear phagocyte system (MPS). However, PEG also inhibits  
14   interactions of nanomedicines with target cancer cells, limiting the effective drug dose that can be  
15   reached within the target tumor. To overcome this dilemma, nanomedicines with stimuli-responsive  
16   cleavable PEG functionality have been developed. These benefit from both long circulation lifetimes en  
17   route to the targeted tumor as well as efficient drug delivery to target cancer cells. In this review, various  
18   stimuli-responsive strategies to dePEGylate nanomedicines within the tumor microenvironment will be  
19   critically reviewed.

20

21   **Keywords:** nanomedicine, cancer, stimuli responsive, dePEGylation, EPR effect

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40    **1. Introduction**

41    In the treatment of cancer, the main challenge is how to deliver cytotoxic drugs to cancer cells while  
42    minimizing off-target toxicity in healthy cells and tissue. Patients currently undergoing cancer  
43    chemotherapy will typically experience debilitating side effects<sup>1</sup> (*e.g.* impaired immune system, nausea,  
44    cardiomyopathy, hair loss), and in many cases, the cumulative lifetime dose of an anti-cancer drug (*e.g.*  
45    doxorubicin; 550 mg/kg) must be limited, irrespective of therapeutic success, to avoid permanent bodily  
46    damage.<sup>2</sup> Efforts have therefore been made to develop nanomedicines capable of delivering drugs  
47    specifically to cancer cells.<sup>3</sup>

48    Over the past 30 years, two clinically effective *targeted* cancer therapies have emerged: antibody-drug  
49    conjugates (ADCs) and nanoparticle-based systems. Currently, 4 ADCs and 7 distinct nanoparticle-based  
50    drug delivery systems (DDS), targeted against a variety of human cancers, have received market  
51    approval.<sup>4, 5</sup> For ADCs, active targeting of cancer cells is achieved through antibody recognition of  
52    (over-)expressed receptors (tumor-associated antigens).<sup>6</sup> Once bound, ADCs are endocytosed, the  
53    conjugated drug released and the cell destroyed. Although effective, ADCs are costly to manufacture,  
54    can elicit adverse immunogenic responses (limiting repeat dosing) and are largely restricted to the  
55    delivery of small molecule (and serum stable) drugs.<sup>7</sup> In the case of nanoparticle-based DDS, drugs are  
56    encapsulated within a self-assembled nanoparticle, hidden and protected from the *in vivo* environment.  
57    Pharmacokinetic (PK) profiles are dictated by the nanoparticle and, in theory, it is possible to deliver  
58    almost any therapeutic cargos, from small molecule drugs to plasmid DNA, to target cells and tissue  
59    within the body. An enormous variety of nanoparticle-based DDS have been reported, however the most  
60    widely investigated, and the majority approved for clinical application, are liposomes.<sup>8</sup> In the targeted  
61    treatment of cancer, all clinically approved nanoparticle-based nanomedicines are liposomes designed to  
62    passively target tumors *via* the enhanced permeability and retention (EPR) effect.<sup>9, 10</sup>

63

64    **1.1    The enhanced permeability and retention (EPR) effect**

65    Following administration to the body, small molecule drugs freely diffuse into tissue and away from the  
66    site of injection. In contrast, intravenously (*i.v.*) injected nanoparticles are restricted to the circulating  
67    blood flow, unable to cross the tightly packed endothelium due to their larger size. For optimal  
68    biodistribution, nanoparticles should be larger than 10 nm in diameter – below which they are filtered  
69    from the body *via* the kidneys<sup>11</sup> – and smaller than 200 nm in diameter – above which they are rapidly  
70    recognized and phagocytosed by blood resident macrophages (principle cells of the mononuclear  
71    phagocyte system, MPS), within the liver and spleen, and are cleared from the body.<sup>12</sup>

72    The EPR effect is a phenomena characterized by the ill-defined ('leaky') vasculature and poor lymphatic  
73    drainage of tumors that arises as a result of rapid angiogenesis (blood vessel growth) within tumor  
74    tissue.<sup>13</sup> Circulating nanoparticles circulating through the tumor vasculature can therefore passively  
75    diffuse across gaps in the endothelium, accumulate within the tumor and remain there for extended

76 periods of time. Once within the tumor, nanoparticle encapsulated drugs either passively diffuse from the  
77 nanoparticle or an endogenous or exogenous stimulus can be exploited to trigger release.

78 To maximize passive targeting of nanomedicines to solid tumors *via* the EPR effect, nanoparticles with  
79 long circulation lifetimes are sought. Put simply, the more times nanoparticles pass through the tumor  
80 vasculature, the more will accumulate there. Care must therefore be taken to minimize drug leakage from  
81 the nanoparticle *en route* to the tumor while ensuring therapeutically relevant concentrations of drugs are  
82 released once there. In the case of liposome-drug formulations, this involves careful choice of lipid  
83 reagents (*e.g.* cholesterol to rigidify fluid lipid membranes) to fine tune drug retention/release profiles  
84 while at the same time maximizing circulation lifetimes.<sup>14</sup>

85

## 86 1.2 Polyethylene glycol (PEG)

87 To achieve long circulation lifetimes, the principal biological barrier a nanoparticle must overcome is  
88 recognition and clearance by cells of the mononuclear phagocyte system (MPS).<sup>15</sup> The principle organ of  
89 the MPS is the liver where hepatic macrophages – Kupffer cells – are highly proficient at recognizing  
90 and removing macromolecular, colloidal and pathogenic waste from circulation.<sup>16, 17</sup> Without any surface  
91 modification, up to 99% of systemically administered nanoparticles are cleared by the liver.<sup>18</sup> In most  
92 cases, it is believed rapid adsorption of blood proteins to the surface of nanoparticles, (a process known  
93 as opsonisation), acts as the recognition beacon for MPS cells.<sup>19</sup> For this reason, sterically shielding  
94 nanoparticle surfaces with biocompatible polymers, such as polyethylene glycol (PEG), has been  
95 effectively employed to minimize opsonisation and prolong blood circulation times of nanoparticles *in*  
96 *vivo*.<sup>20</sup>

97 PEG is a synthetic polymer of repeating ethylene glycol units. Used as a reagent or additive in a wide  
98 range of biological, chemical and industrial settings,<sup>21, 22</sup> it is commercially available in a range of  
99 geometries (linear, branched, star, comb), molecular weights (from 300 Da – 6-7 repeating units – up to  
100 10 MDa - >200,000 repeating units) and can be easily functionalized. PEylation of nanoparticle  
101 surfaces has been shown to decrease serum protein adsorption, reduce nanoparticle uptake in the liver  
102 and prolong circulation lifetimes.<sup>23</sup> Recently, reports have emerged to suggest PEG can elicit an  
103 immunogenic response in mammals.<sup>24</sup> However, the extent of this response, caused by binding of  
104 anti-PEG antibodies, remains unclear.<sup>25</sup> PEG remains an FDA approved polymer and is still the most  
105 widely used polymeric coating of nanomedicines, both in academic and industrial research. In terms of  
106 cancer nanomedicines, PEGylated liposomal-doxorubicin (Doxil®) has been used clinically for over 20  
107 years in the treatment of select breast and ovarian cancers, multiple myeloma and AIDS-related Kaposi's  
108 sarcoma.<sup>22</sup>

109

110    **1.3    The PEG dilemma**

111    While PEGylation prolongs circulation lifetimes, it also limits the cellular uptake of nanoparticles and  
112    therefore effective drug delivery to target cancer cells.<sup>26</sup> This so-called ‘PEG dilemma’ has proved a  
113    major obstacle in the effective delivery of therapeutic cargos to cancer cells, particularly those that must  
114    be actively transported across the target cellular membrane (*e.g.* proteins and oligonucleotides).<sup>27</sup> For  
115    instance, in the delivery of oligonucleotides (ODNs) or small interfering RNAs (siRNAs), significantly  
116    lower transfection/transduction efficiencies were observed for PEGylated vs. non-PEGylated DDS.<sup>28</sup> To  
117    overcome this dilemma, strategies have been proposed to trigger the *extracellular* shedding of PEG (*i.e.*  
118    dePEGylation) from a nanoparticle surface upon reaching the target tumor. This leads to one of three  
119    scenarios (Figure 1): 1) rupture of the nanoparticle and extracellular drug release; 2) cellular uptake  
120    (endocytosis) of the intact nanoparticle-drug complex or 3) in the case of liposomes, fusion with the  
121    target cellular membrane and drug release directly to the cell cytoplasm, crucially avoiding degradative  
122    endocytotic liposome uptake.

123    In a significant number of reported dePEGylation strategies, it is required that PEGylated nanoparticles  
124    are first taken up by target cancer cells, whereupon the low pH, reductive and protease-rich environment  
125    of the late endosome/lysosome can be effectively exploited to trigger *intracellular* dePEGylation and  
126    drug release. However, these systems *do not* overcome the “PEG dilemma” and the very limited uptake  
127    of PEGylated nanoparticles remains a major drawback. As such, these systems will not be further  
128    discussed in this review but are included in the comprehensive summary of dePEGylation strategies  
129    presented in Table 1.

130    For strategies involving *extracellular* dePEGylation within the target tumor, a key difference is whether  
131    dePEGylation causes destabilization of the nanocarrier and *extracellular* drug release (*i.e.* burst release),  
132    or intact nanocarrier internalization by target cancer cells and *intracellular* drug release. In the case of  
133    *extracellular* drug release, only drugs able to passively diffuse (or be actively transported) across target  
134    cancer cell membranes (*e.g.* membrane permeable doxorubicin) can be used. In the case of *intracellular*  
135    drug release, the delivery of membrane impermeable therapeutics (*e.g.* proteins, oligonucleotides) is  
136    possible. In either scenario, it is essential cancer cells are exposed to therapeutically relevant doses of  
137    cytotoxic drugs if improved therapeutic indices are to be achieved.

138

139    **2. Physical dePEGylation**

140    Two physical approaches to dePEGylate nanoparticle surfaces within target tissues have been  
141    investigated. The first, most relevant for liposomal nanomedicines, relies on the exchange of PEGylated  
142    lipids from a liposome membrane to a target membrane sink (*e.g.* target cancer cell membranes).<sup>29</sup> Here,  
143    the rate at which exchange occurs is heavily dependent on the lipid anchor tethering PEG to the liposome  
144    membrane (*i.e.* how strongly it is held within the liposome membrane).<sup>30</sup> The length and saturation of  
145    (phospho)lipid fatty acid (FA) chains determines both the thickness and rigidity of a lipid membrane. FA

146 chain lengths within biological membranes typically vary between C12 and C30 – the number of carbon  
147 atoms.<sup>31</sup> FA chains can be saturated (no double bonds) or unsaturated (1 or more double bond). Saturated  
148 FAs pack closely together to form rigid lipid membranes (gel state), whereas unsaturated FAs loosely  
149 pack to form fluid membranes liquid crystalline state).<sup>32</sup> In addition, the shorter the FA chains, the more  
150 fluid the membrane. This is reflected in the liquid crystalline-to-gel transition temperatures ( $T_m$ ) of  
151 individual (phospho)lipids.

152 In a study of three different lipid-PEG conjugates, no lipid-PEG exchange was observed for long chain,  
153 saturated lipid anchors 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG, C18:0 – 2 x 18  
154 carbon FA chain; no double bonds) whereas exchange occurred in the time frame of hours for shorter  
155 saturated lipids 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE-PEG; C14:0) or long chain,  
156 unsaturated lipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE-PEG; C18:1 – 2 x 18 carbon  
157 FA chain; each 1 double bond,  $\omega$ 9).<sup>33</sup> This time frame enabled efficient accumulation of liposomes in  
158 tumor sites *via* the EPR effect (prior to dePEGylation) coupled with increased cellular uptake within the  
159 tumor (following dePEGylation). Conversely, a similar study found that only in the case of DSPE-PEG  
160 were circulation times prolonged enough to see efficient passive accumulation of nanoparticles within  
161 the tumor.<sup>34</sup> These conflicting results highlight the fine balance required to achieve efficient passive  
162 accumulation within target tumors and subsequent dePEGylation *via* physical desorption of lipid-PEG  
163 reagents. The propensity for non-specific PEG exchange with biological membranes *in vivo*, prior to  
164 reaching the target tumor, has likely limited the widespread application of these approaches.

165 The second physical approach relies on non-covalent adsorption of PEG to a nanoparticle surface.<sup>35-39</sup>  
166 For example, carboxylate-functionalized PEG adsorbed to a cationic nanoparticle surface.<sup>37</sup> In this case,  
167 partial protonation of carboxylate groups within the acidic (pH 6.5-7) extracellular tumor  
168 microenvironment leads to dePEGylation and subsequent cellular nanoparticle uptake. While this  
169 approach is conceptually simple, the stability of the absorbed PEG corona in serum and the propensity of  
170 premature dePEGylation under physiological conditions (*e.g.* high salt) and/or through competition from  
171 other serum components has likely limited the widespread investigation of this approach.

172

### 173 3. Chemical dePEGylation strategies

174 By far the most common method to achieve *extracellular* dePEGylation of nanoparticle surfaces, within  
175 the tumor microenvironment, is through chemical approaches. In these cases, PEG is grafted to the  
176 nanoparticle *via* a stimuli-responsive covalent chemical bond (Table 1 and 2).<sup>40</sup> Stimuli can be both  
177 endogenous and exogenous. In the case of endogenous stimuli, intrinsic differences in the  
178 pathophysiology of tumor and healthy tissues are exploited, namely the low pH,<sup>41</sup> reducing<sup>42</sup> and matrix  
179 metalloprotease (MMP)-rich environment<sup>43</sup> of certain solid tumors. Exogenous stimuli, including light  
180 and heat, have the benefit of being under complete user control in both time and space.<sup>44</sup> In a clinical  
181 setting however, these approaches rely on the ability to efficiently deliver stimuli (*e.g.* light) to tissues

182 often deep within the body. The various stimuli-responsive chemistries commonly used in both the *intra*-  
183 and *extracellular* dePEGylation of nanoparticles are summarized in Table 1 and 2.

184

185 **3.1 pH-sensitive dePEGylation**

186 The mildly acidic (pH 6.5-7.2) extracellular environment of hypoxic tumors – a result of increased  
187 glucose catabolism and efflux of H<sup>+</sup> by cancer cells – has been exploited to trigger extracellular  
188 dePEGylation of nanoparticle surfaces.<sup>45</sup> For this, chemical functionalities stable at physiological pH (pH  
189 7.4) but labile at lower pH are required. The most commonly used acid labile chemical groups are vinyl  
190 ethers,<sup>46-50</sup> hydrazones,<sup>51-61</sup> acetals,<sup>62-69</sup> β-thiopropionates,<sup>70</sup> ortho esters<sup>71-73</sup> and benzoic imines<sup>74-82</sup>. Here  
191 however, it is important to differentiate between the mildly acidic *extracellular* pH within the tumor  
192 microenvironment (pH 6.5-7) and the strongly acidic *intracellular* pH within late endosomes/lysosomes  
193 (pH 4.5-5.5) and to stress that optimal sensitivity (and subsequent dePEGylation efficiency) of these  
194 acid-labile functionalities is typically at pH 5-5.5. Therefore all these pH-sensitive systems demonstrate  
195 inefficient/sluggish acidolytic dePEGylation within the *extracellular* tumor microenvironment. This can  
196 be exploited to achieve prolonged and sustained drug release within the tumor and/or partial  
197 dePEGylation may still generate the desired outcome. For example, Gu *et al.* reported pH dependent  
198 dePEGylation of polycationic micelles through grafting of PEG, *via* benzoic imine linkages, to  
199 poly-L-lysine(PLL)/cholic acid co-polymers.<sup>82</sup> By measuring changes in surface charge (zeta potential),  
200 the authors were able to show colloidal stability at physiological pH as well as increasing rates of  
201 dePEGylation with decreasing pH (complete acidolysis at pH 5.5 within 10 min). At pH 6.5-7 (*i.e.* pH of  
202 the extracellular tumor microenvironment) only partial dePEGylation was observed, however this was  
203 accompanied by a significant increase in hemolytic activity suggesting partial dePEGylation was  
204 sufficient to endow these particles with the desired function. As this system was not tested in cancer  
205 models *in vivo*, it remains to be seen whether this slow rate of acidolysis will adversely affect function  
206 and efficacy. Indeed, the individual successes of pH responsive dePEGylation systems ultimately  
207 depends on the ability to deliver therapeutically relevant drug doses to cancer cells above and beyond  
208 those of the administered free drug alone. It is worth noting, however, these technologies – as with any  
209 system exploiting endogenous stimuli – will likely demonstrate significant variations in efficacy due to  
210 patient-to-patient heterogeneity of tumor pathologies.<sup>83, 84</sup>

211

212 **3.2 Redox-sensitive dePEGylation**

213 Glutathione (GSH), is an abundant reducing agent (2-10 mM) in most mammalian cells, including cancer  
214 cells.<sup>85</sup> Extracellular GSH concentrations in healthy tissue are approximately 1000x lower (2–20 μM),<sup>86</sup>  
215 however this value can increase up to 4-fold (4-80 μM) within the tumor microenvironment.<sup>87</sup> There are  
216 conflicting reports as to whether this small differential in extracellular GSH concentrations can indeed be  
217 exploited to trigger *extracellular* dePEGylation. While a small number of studies report (partial)

218 extracellular cleavage of disulfide linked PEG constructs within the tumor microenvironment,<sup>88-92</sup> most  
219 exploit GSH as an *intracellular* trigger only.<sup>93-134</sup> In these cases, the very large differential between *extra-*  
220 and *intracellular* GSH concentrations is a readily exploitable endogenous trigger. Indeed, for systems  
221 designed to exploit *intracellular* GSH levels, *extracellular* stability (*i.e.* very limited reduction) of  
222 disulfide-PEG constructs is often reported as a key feature in maintaining colloidal stability of  
223 nanoparticles in circulation and *en route* to the target tumor. In our critical opinion, exploiting the  
224 marginally elevated *extracellular* GSH levels of the tumor microenvironment is an ineffective strategy to  
225 overcome the ‘PEG dilemma’.

226

### 227 **3.3 Protease-sensitive dePEGylation**

228 Within the tumor microenvironment, there are high levels of *extracellular*  
229 matrix metalloproteinases (MMPs). These lytic enzymes are secreted at high levels by tumor cells to  
230 degrade the extracellular matrix (ECM) and aid cancer cell migration.<sup>135, 136</sup> Short peptides containing  
231 enzyme-consensus sequences, linking PEG to a nanoparticle surface, have been effectively used to  
232 dePEGylate nanoparticles within the tumor microenvironment.<sup>137-161</sup> Torchilin *et al.* have reported two  
233 elegant examples of MMP-triggered dePEGylation. The first employed a multifunctional liposomal  
234 formulation comprising longer, MMP-cleavable lipid-PEG<sub>3400</sub> constructs and shorter, non-cleavable  
235 TAT-functionalised lipid-PEG<sub>2000</sub> constructs.<sup>141</sup> In the absence of MMPs, longer PEG<sub>3400</sub> chains  
236 effectively shielded the cell penetrating function of the underlying TAT peptide and liposomes were  
237 sparingly taken up by cells. Upon MMP-mediated dePEGylation however, the newly revealed  
238 TAT-functionalised liposomes were avidly taken up by 4T1 breast cancer cells. Going one step further,  
239 the same group reported a similar strategy of exploiting MMP-mediated dePEGylation to reveal newly  
240 functional drug polymer micelles.<sup>146</sup> Crucially in this approach, dePEGylation did not destroy the  
241 integrity of the underlying drug-filled micelle leading to efficient stimuli responsive, *intracellular* drug  
242 delivery to cancer cells, as demonstrated in mice models.

243 It is worth noting here that both cathepsin B (protease)<sup>162</sup> and esterases<sup>138</sup> have also been exploited to  
244 trigger dePEGylation of nanomedicines. However, cathepsin B is only found at high levels within  
245 (*intracellular*) cancer cells, while esterases are widely distributed in plasma and healthy tissues and not  
246 therefore specific to the tumor microenvironment. In our opinion, MMP-mediated dePEGylation of  
247 nanoparticles within the tumor microenvironment represents the most selective and efficient strategy to  
248 enhance the efficacy of cancer nanomedicines exploiting *endogenous* stimulus.

249

### 250 **3.4 Light-sensitive dePEGylation**

251 Photolabile chemical bonds have been extensively used, in both chemistry and biological contexts, to  
252 precisely control where and when new functionality is revealed. Unlike endogenous stimuli such as pH,

253 redox and enzymatic cleavage, the application of light can be precisely controlled in both time, space and  
254 intensity (*i.e.* is user defined) and requires no other reactive species (other than, in some cases, water). In  
255 addition, photolysis is generally rapid (few seconds, pulsed laser), quantitative and clean.

256 For light triggered dePEGylation of potential cancer nanomedicnes, *o*-nitrobenzyl (*o*-Nb),<sup>63, 163-166</sup>  
257 platinum-azide complexes<sup>167</sup> and azobenze<sup>168</sup> functionalities have all been explored.<sup>169</sup> In the case of  
258 *o*-Nb functionalities, non-hydrolytic photolysis proceeds through a cyclic intermediate followed by the  
259 release of the desired alcohol and a nitroso by-product.<sup>170</sup> To increase biological compatibility, methoxy  
260 substitution of the aryl ring results in reduced toxicity of nitroso byproducts.<sup>171</sup> We have recently  
261 reported two separate strategies in which light triggered dePEGylation was successfully used to initiate  
262 efficient drug delivery to target cancer cells. In the first example, we created 100 nm, loose core shell  
263 micelles composed exclusively of photolabile doxorubicin-PEG<sub>2000</sub> reagents.<sup>165</sup> In the absence of light,  
264 micelles were stable, non-toxic (*i.e.* not taken up by cells *in vitro*) and no doxorubicin release was  
265 observed over time. Upon light (365 nm) activation triggered dePEGylation, micelle destabilisation and  
266 subsequent burst drug release resulted in *in vitro* cytotoxicity comparable to free doxorubicin. In  
267 addition, we were able to demonstrate precise spatiotemporal control of doxorubicin delivery to cells *in*  
268 *vitro* through light templated activation. We are currently assessing this system *in vivo* to determine  
269 circulation lifetimes and tumor accumulation of PEGylated doxorubicin prodrug micelles prior to light  
270 triggered dePEGylation.

271 In the second example, light triggered dePEGylation was used to precisely control, in time and space, the  
272 function of a simplified membrane fusion system. This system comprises two complementary peptides –  
273 peptide E and K – displayed from opposing lipid membranes (either liposome-liposome or  
274 liposome-cell).<sup>163</sup> In this case, PEGylation (*via* a photolabile cholesterol-PEG construct) of one lipid  
275 membrane effectively shielded the interaction between complementary peptides. However, upon light  
276 triggered dePEGylation regain of fusion function was instantaneous. We have subsequently shown our  
277 simplified membrane fusion system can be used to selectively deliver liposome-encapsulated cargos, *via*  
278 membrane fusion, to target (xenografted) cancer cells *in vivo* (zebrafish larvae).<sup>172</sup> Extending this  
279 approach to include light triggered dePEGylation, to enable precise user control of drug delivery, is the  
280 subject of current investigations in the group.

281 The use of light does, of course, raise valid concerns going forward into the clinic. In all reported  
282 examples of light triggered dePEGylation, systems are most sensitive to high energy UV-A light (<400  
283 nm). Short wavelength UV light suffers from poor tissue penetration (100-200 μm) and, following  
284 prolonged exposure, can elicit significant photocytotoxicity.<sup>173</sup> Only for polymeric systems containing  
285 platinum-azide complexes<sup>167</sup> was photolytic dePEGylation investigated using visible light irradiation.  
286 Here, decreasing photolytic efficiency correlated with longer light wavelengths. Here however, it is  
287 important to note that photodynamic therapies,<sup>174</sup> combining chemical photosensitizers and light  
288 activation, are already widely used in the clinic to treat a range of medical conditions, including acne,  
289 atherosclerosis and cancer.<sup>175</sup> Furthermore, advances in fibre optic technologies (to deliver UV light deep

290 within tissue),<sup>176</sup> the development of photolabile chemical bonds sensitive to longer wavelength light<sup>177</sup>  
291 and the optimization of photosensitive chemical functionality to minimize light exposure, will only  
292 further the clinical applicability of light. One promising development has been photolabile chemical  
293 groups sensitive to two photon light,<sup>178</sup> to not only increasing tissue penetration (>1 cm) of light and  
294 minimising induced photocytotoxicity but, by restricting light activation to the focal point of two photon  
295 beams, enabling activation volumes in patients of <1 femtolitre. In this vein, we and others have also  
296 shown it is possible to cleave *o*-Nb groups using 2-photon light.<sup>179</sup> There are currently no examples of  
297 responsive dePEGylation of nanoparticles using alternative external stimuli (*e.g.* heat or ultrasound).

298

299 **4. Conclusion**

300 Stimuli-responsive dePEGylation is a proven strategy to increase the efficacy of cancer nanomedicines  
301 passively targeting solid tumors *via* the EPR effect. This approach has the dual advantage of both  
302 extended circulation lifetimes of PEGylated nanoparticles (to enhance passive targeting efficiency to  
303 tumors) as well as enhanced drug delivery profiles of non-PEGylated (or ruptured) nanoparticles within  
304 the tumor microenvironment. To achieve maximal effect, nanomedicines must remain PEGylated *en*  
305 *route* to the tumor (*i.e.* are serum stable) and be efficiently dePEGylated within the *extracellular* tumor  
306 microenvironment. Given the very low cellular uptake of PEGylated nanoparticles, strategies that report  
307 stimuli-responsive *intracellular* dePEGylation should not be considered effective. In our view, the most  
308 promising stimuli-responsive nanomedicines to date have exploited the MMP-rich microenvironment of  
309 solid tumors to trigger targeted and *extracellular* dePEGylation. However, by exploiting endogenous  
310 pathophysiological differences between healthy and diseased tissue, such as differences in MMP  
311 concentrations, the efficacy of these stimuli-responsive systems in patients will likely vary due to  
312 patient-to-tumor tumor heterogeneity.<sup>83</sup> In contrast, dePEGylation triggered by external stimuli, such as  
313 light, is exclusively determined by the user. While these approaches negate potential differences in  
314 efficacy driven by tumor heterogeneity, the current technological limitations of delivering external  
315 stimuli to site specific locations in patients remains a major drawback. However, the continued advance  
316 and optimisation of fibre-optic technologies as well more advanced photolabile chemical groups will  
317 only promote further the future application of light triggered cancer nanomedicines.

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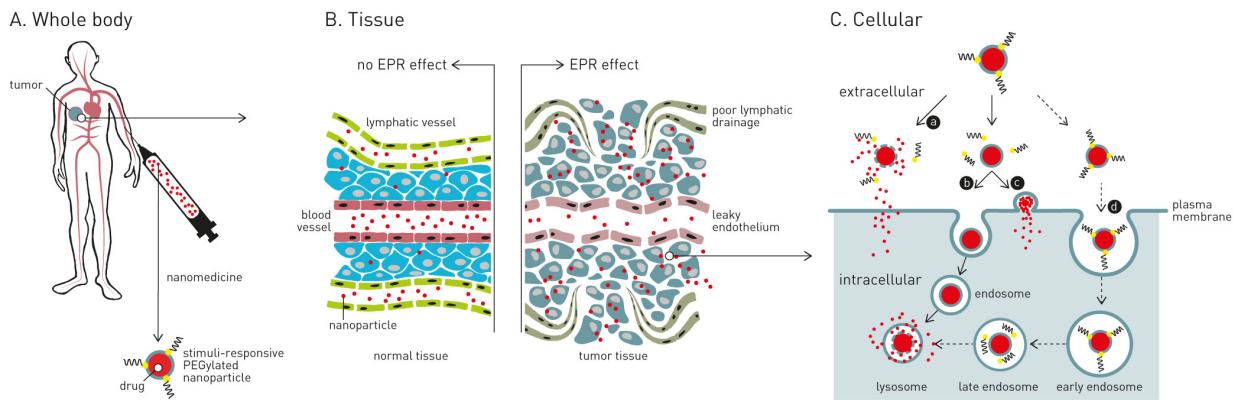
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325 **Figure 1.** Following passive targeting of solid tumors *via* the enhanced permeability and retention (EPR)  
326 effect, stimuli-responsive dePEGylation of cancer nanomedicines can lead to various routes of enhanced  
327 drug delivery: **route a** – extracellular dePEGylation, nanocarrier rupture and extracellular drug delivery;  
328 **route b** – extracellular dePEGylation, endocytotic nanocarrier uptake and intracellular drug delivery;  
329 **route c** – extracellular dePEGylation, nanocarrier fusion with cancer cell membrane and direct cytosolic  
330 drug delivery (most relevant for liposomal nanomedicines); **route d\*** – endocytotic nanocarrier uptake,  
331 intracellular dePEGylation and intracellular drug delivery. \* this route *does not* overcome the “PEG  
332 dilemma” and the very limited uptake of PEGylated nanoparticles is a major drawback of these systems.

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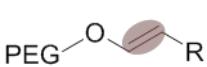
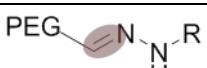
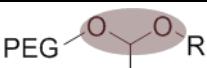
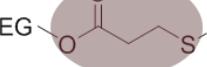
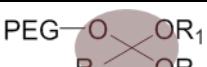
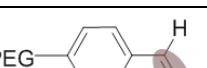
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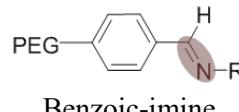
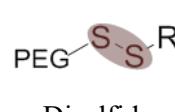
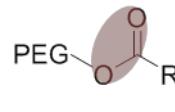
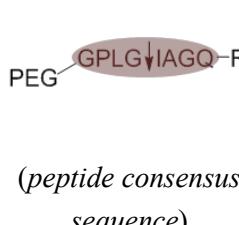
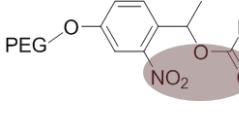
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Site of dePEGylation within tumor	Stimulus	Example chemical structure	Nanocarrier	Drug release [refs]	
				Intracellular	Extracellular
Intracellular*	Low pH Late endosome (pH <6.5) Lysosome (pH 5.5-6.5)	PEG-O-  R Vinylether	Liposomes	[46-49]	
		 R Hydrazone	Micelles	[50]	
		 R Acetal	Liposomes	[52-54]	
			Micelles	[55-61]	
		 R β-thiopropionate	Liposomes	[62]	
			Micelles	[63-69]	
		 R Ortho ester	Liposomes	[70]	
		 R Benzoic-imine	Micelles	[71-73]	
		 R	Liposomes	[74-76]	
			Polymersomes	[93]	
			Micelles	[94, 95]	
			Graphene Oxide	[96-121, 133]	
			Mesoporous silica nanoparticles (MSN)	[122-124]	
			Magnetic nanoparticles	[125-132]	
Enzymatic	Cathepsin B	 R (peptide consensus sequence)	Liposomes	[162]	

349 **Table 1.** Various stimuli responsive chemical functionality used to trigger intracellular dePEGylation of  
350 cancer nanomedicines within the tumor microenvironment. \*given the very limited uptake of PEGylated  
351 nanoparticles, systems reliant on intracellular triggers do not overcome the ‘PEG dilemma’ and are not  
352 further discussed in this review.

Site of dePEGylation within tumor	Stimulus	Example chemical structure	Nanocarrier	Drug release [refs]	
				Intracellular	Extracellular
Extracellular	<b>Low pH</b> (pH 6.5-7)	 Benzoic-imine	Polymeric Nanoparticles	[77]	
			Magnetic nanoparticles		[78-80]
			Micelles	[81]	[82]
	<b>Reduction</b> Glutathione (GSH) (4-80 µM)	 Disulfide	Liposomes		[88-92]
	<b>Enzymatic (non-specific)</b> Esterase		Liposomes	[137, 138]	
	<b>Enzymatic (specific)</b> Matrix metallo-proteinases (MMPs)	 (peptide consensus sequence)	Liposomes	[139-145]	
			Quantum dots (QDs)	[157]	
			Polymersomes	[158]	
			Magnetic nanoparticles	[159]	
			Micelles	[146-156]	[160, 161]
	<b>External Light</b>		Liposomes	[163]	
			MSN	[164]	
			Micelles	[168]	[63, 165-167, 169]

**Table 2.** Various stimuli responsive chemical functionality used to trigger extracellular dePEGylation of cancer nanomedicines within the tumor microenvironment.

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