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1 **Maleimide-functionalised PLGA-PEG nanoparticles as** 2 **mucoadhesive carriers for intravesical drug delivery**

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17 **Abstract**

18 Low permeability of the urinary bladder epithelium, poor retention of the chemotherapeutic agents due
19 to dilution and periodic urine voiding as well as intermittent catheterisations are the major limitations of
20 intravesical drug delivery used in the treatment of bladder cancer. In this work, maleimide-functionalised
21 poly(lactide-*co*-glycolide)-*block*-poly(ethylene glycol) (PLGA-PEG-Mal) nanoparticles were
22 developed. Their physicochemical characteristics, including morphology, architecture and molecular
23 parameters have been investigated by means of dynamic light scattering, transmission electron
24 microscopy and small-angle neutron scattering techniques. It was established that the size of
25 nanoparticles was dependent on the solvent used in their preparation and molecular weight of PEG, for
26 example, 105 ± 1 nm and 68 ± 1 nm particles were formed from PLGA_{20K}-PEG_{5K} in dimethyl sulfoxide
27 and acetone, respectively. PLGA-PEG-Mal nanoparticles were explored as mucoadhesive formulations
28 for drug delivery to the urinary bladder. The retention of fluorescein-loaded nanoparticles on freshly
29 excised lamb bladder mucosa *in vitro* was evaluated and assessed using a flow-through fluorescence
30 technique and Wash Out₅₀ (WO₅₀) quantitative method. PLGA-PEG-Mal nanoparticles (NPs) exhibited
31 greater retention on urinary bladder mucosa (WO₅₀ = 15 mL) compared to maleimide-free NPs (WO₅₀ =
32 5 mL). The assessment of the biocompatibility of PEG-Mal using the slug mucosal irritation test revealed
33 that these materials are non-irritant to mucosal surfaces.

34 **Keywords:** urinary bladder, intravesical drug delivery, PLGA-PEG, maleimide, nanoparticles, small-
35 angle neutron scattering, slug mucosal irritation test, mucoadhesion, Wash Out₅₀ (WO₅₀).

36

37 1. Introduction

38 Mucoadhesion can be described as the ability of materials to adhere to mucosal membranes for
39 extended periods of time. Transmucosal drug administration is currently employed in ocular
40 (conjunctival and corneal), nasal, pulmonary, oromucosal (buccal and sublingual), gastrointestinal,
41 rectal, vaginal and intravesical drug delivery, and it offers an alternative to injections [1–6].
42 Transmucosal drug delivery provides numerous advantages, including (i) prolonged residence time of a
43 dosage form and high density of blood vessels on mucosal surfaces ensure more efficient and rapid drug
44 absorption; (ii) non-oral drug administration allows avoiding its potential degradation in the stomach and
45 prevents hepatic first-pass metabolism; (iii) ease of drug administration and the possibility for quick
46 termination of a therapy resulting in improved patient compliance; (iv) possibility of targeting particular
47 body sites and tissues due to local administration; and (v) reduced administration frequency.
48 Mucoadhesive dosage forms could be highly beneficial for local drug administration to treat posterior
49 segment diseases of the eye [7,8], neurological disorders (*via* intranasal administration) [9,10], and
50 genitourinary tract dysfunctions [11,12].

51 Intravesical drug delivery (IDD) refers to a direct administration of active pharmaceutical
52 ingredients into the urinary bladder using a catheter inserted through the urethra. This technique is
53 generally used to treat bladder-related disorders, such as bladder cancer (BC) and interstitial cystitis [13–
54 15]. However, the efficiency of this route of drug administration is limited due to dilution and wash out
55 during periodical micturition resulting in poor retention of instilled therapeutic agents. Additionally, the
56 need for frequent catheterisations with potential risks of irritation, inflammatory reactions and infections
57 makes this procedure rather unpleasant for patients [16].

58 Mucoadhesive materials have the potential to improve the efficacy of IDD by prolonging the drug
59 residence in the bladder. First generation (conventional) mucoadhesive materials are traditionally used
60 as matrixes in many formulations for transmucosal drug delivery. These include hydrophilic polymers
61 of natural and synthetic origin, such as chitosan, carbopols and cellulose derivatives [17–21]. The

62 adhesion of these macromolecules is due to their ability to interact with glycosylaminoglycans/mucins
63 present on mucosal surfaces through non-covalent interactions such as hydrogen bonding, electrostatic
64 attraction and chain entanglement.

65 Andreas Bernkop-Schnürch [22] pioneered the use of thiolated polymers (thiomers) as the second
66 generation of mucoadhesives. Well-established water-soluble polymers were modified with thiol
67 functional groups using different chemical approaches to greatly enhance their mucoadhesive properties
68 [23–25]. A few studies reported the use of thiolated mucoadhesives for IDD. For example, thiol-
69 functionalised chitosan nanoparticles have been used for IDD in an *in vitro* study using porcine urinary
70 bladders [26,27]. The ability of these thiolated mucoadhesives to stay adhered on mucosal surfaces is
71 due to the formation of inter-disulphide bridges *via* covalent interaction with cysteine residues present in
72 mucins.

73 Recently, our group has demonstrated that polymeric nanogels [28] and PEGylated liposomes [29]
74 functionalised with maleimide groups exhibited excellent mucoadhesive properties to bovine
75 conjunctival tissues and freshly excised porcine bladder mucosa, respectively. The ability of these
76 formulations to retain on mucosal tissues, evaluated using a flow-through method, was found to be
77 comparable to a well-known mucoadhesive chitosan. The excellent mucoadhesive performance of these
78 novel advanced formulations is attributed to their ability to form covalent linkages with thiol groups
79 present in the mucus *via* Michael-type addition reactions. Later, other researchers have also reported the
80 development of alginate- and chitosan-based mucoadhesives functionalised with maleimide groups and
81 demonstrated their superior retention/adhesion on/to mucosal surfaces compared to unmodified
82 polysaccharides [30, 31]. All the maleimide-functionalised systems reported so far [28–31] due to their
83 hydrophilic nature were only suitable for formulation of water-soluble drugs. There is also a strong need
84 in the development of mucoadhesive drug delivery systems capable of incorporating poorly-soluble
85 drugs, which will require the use of less polar polymers for their development.

86 Poly(lactide-*co*-glycolide)-*block*-poly(ethylene glycol) (PLGA-PEG), a block copolymer
87 approved by the US Food and Drug Administration, has been widely employed in controlled release
88 formulations and tissue engineering applications due to its safety, low toxicity, low immunogenicity,
89 high cytocompatibility and biodegradability [32]. PLGA-PEG could easily be self-assembled to micelles
90 or nanoparticles by either simple single emulsion (oil-in-water) or double emulsion solvent evaporation
91 (water/oil/water) techniques [33,34]. For instance, Bazylińska et al. [35] described the influence of
92 preparation parameters, the type of polymer and active cargo on the colloidal and biological stability of
93 final nano-carriers for further *in vitro* and *in vivo* applications. Pei et al. developed the application of pH-
94 sensitive intravenous PLGA-PEG formulations to deliver vancomycin in the therapy against intracellular
95 pathogens [36]. PLGA-PEG nanoparticles functionalised with maleimide groups were also recently
96 reported as carriers for drug delivery. Their maleimide-functionalised surface was used for further
97 modification with monoclonal antibody [37] and cell penetrating peptides [38].

98 In the present work, we report the design of maleimide-functionalised PLGA-PEG nanoparticles
99 as potential mucoadhesive formulations for IDD to urinary bladder and demonstrate that surface
100 maleimide groups could significantly enhance mucoadhesive properties of these materials. Four types of
101 nanoparticles were developed using PLGA-PEG containing 3 and 5 kDa PEG with and without
102 maleimide terminal groups. The structural features of these nanoparticles were studied using small-angle
103 neutron scattering, dynamic light scattering and transmission electron microscopy. The hydrophobic
104 nature of PLGA-based core of the nanoparticles provided an excellent opportunity for formulating poorly
105 water-soluble compound as a model drug. These nanoparticles were loaded with lipophilic fluorescein
106 and their retention on urinary bladder mucosa was studied *in vitro*. Slug mucosal irritation *in vivo* test
107 was used to assess the biocompatibility of PEG-Mal present on the surface of these nanoparticles.

108

2. Materials and methods

2.1. Materials

Four different di-block copolymers of poly(lactide-*co*-glycolide)-poly(ethylene glycol) ((methoxy-terminated: Mw 20,000 : 3,000 Da, PDI 1.30 (PLGA_{20K}-PEG_{3K}) and Mw 20,000 : 5,000 Da, PDI 1.65 (PLGA_{20K}-PEG_{5K}); maleimide-functionalised: Mw 20,000 : 3,400 Da, PDI 2.70 (PLGA_{20K}-PEG_{3K}-Mal) and Mw 20,000 : 5,000 Da, PDI 2.42 (PLGA_{20K}-PEG_{5K}-Mal)) were purchased from PolySciTech[®] Akina Inc. (West Lafayette, IN, USA; catalogue numbers: AK101, AK037, AI109, AI020). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Loughborough, UK), acetone and deuterium oxide (D₂O), benzalkonium chloride (BAC), poly(ethylene glycol) methyl ether (PEG; average Mn 5,000 Da), methoxypolyethylene glycol maleimide (PEG-Mal; average Mn 5,000 Da) and phosphate buffered saline tablets (PBS) were purchased from Sigma-Aldrich (Gillingham, UK). 6-Maleimidohexanoic acid was purchased from Alfa Aesar (Heysham, UK). Fluorescein was purchased from Acros Organics (Geel, Belgium). All other chemicals were of analytical grade and used as received.

2.2. Preparation of nanoparticles

Empty and fluorescein-loaded PLGA-PEG nanoparticles were prepared *via* one-step nanoprecipitation (single emulsion) technique followed by dialysis. In brief, 20 mg of polymers and 1 mg fluorescein were dissolved in 1 mL of DMSO or acetone to form organic phase. The organic phase was then added to 30 mL of deionised water dropwise under constant stirring to form a colloidal suspension, which was allowed to stir gently for 1 h. Finally, this suspension was purified by dialysis against deionised water (5L; 4 changes) using a dialysis membrane tube (molecular weight cut-off 12–14 kDa; Medicell Membranes Ltd., UK) to remove residual solvent, filtered using 0.22 µm Minisart[®] syringe filters and stored in a fridge for further use.

2.3. Particle size and zeta potential analysis

132 The size of fluorescein-free PLGA-PEG nanoparticles, their polydispersity index (PDI) and zeta-
133 potential values were determined using dynamic light scattering (DLS) at a scattering angle of 173° with
134 a Zetasizer Nano-NS (Malvern Instruments, UK). Each nanoparticle dispersion was diluted 100-fold with
135 ultra-purified water. Refractive index of 1.59 and absorbance of 0.01 were used in all measurements.
136 Each sample was analysed three times at 25 °C and the Z-average mean \pm standard deviation values were
137 calculated.

138 **2.4. Transmission electron microscopy (TEM)**

139 Nanoparticles were imaged using a JEOL 2100 Plus TEM operated at an acceleration voltage of
140 200 kV. Specimens were prepared by pipetting a drop of purified nanoparticle suspension (about 0.5
141 mg/mL) onto a parafilm. A glow-discharged holey carbon film-coated 400-mesh copper grid was then
142 placed onto the drop with “carbon” side and left in contact with the sample for 60 sec. The excess solution
143 was removed by blotting with a filter paper. Then, a drop of 2% (w/v) uranyl acetate (UA) solution was
144 deposited onto the parafilm and the grid remained in contact with “carbon” side with UA for another 60
145 sec. The excess stain was removed by dabbing similarly as above and the sample was left to dry in air
146 prior to TEM characterisation. This sample preparation technique was previously reported to give good
147 quality of images for PLGA-PEG nanoparticles [39].

148 **2.5. Small-Angle Neutron Scattering (SANS) study**

149 PLGA-PEG nanoparticles were prepared as described above. Briefly, 10 mg of polymers were
150 dissolved in 1 mL of organic solvent (acetone or DMSO). This solution was then added to 20 mL of D₂O
151 dropwise under continuous stirring to form suspension of nanoparticles, which was then allowed to stir
152 gently for an additional 20 min. Finally, 2 mL of aliquot was aspirated from the suspension and dialysed
153 using a Spectra-Por[®] Float-A-lyzer[®] G2 dialysis membrane, with a molecular weight cut-off 3.5–5 kDa,
154 against D₂O to remove residual solvent, filtered using 0.22 μ m Minisart[®] syringe filters and stored in a
155 fridge prior to SANS studies.

156 SANS experiments were performed at MLZ (Garching, Germany) on KWS-2 instrument [40].
 157 Measurements were made on a ^3He tubes array detector (144 tubes, pixel size 8 mm) using a non-
 158 polarised, monochromatic (wavelength λ set by a velocity selector) incident neutron beam collimated
 159 with rectangular apertures for two sample-to-detector distances, namely 2, 8, and 20 m ($\lambda = 0.6$ nm).
 160 With this setup, the investigated q -range was 0.015 nm^{-1} to 4.6 nm^{-1} . In all cases, the two-dimensional
 161 scattering patterns were isotropic and were azimuthally averaged, resulting in the dependence of the
 162 scattered intensity $I_s(q)$ on the momentum transfer $q = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle. The
 163 curves were corrected for background scattering from the empty cell and for detector efficiency. Hellma[®]
 164 Analytics Suprasil[®] 300 high precision quartz cells of 1 and 2 mm thickness were used for experiments.
 165 SANS experiments were performed in D_2O solution. The D_2O solution was measured and properly
 166 subtracted. The concentration of nanoparticles used in SANS measurements was 0.5 mg/mL.

167 **2.6. SANS data fitting**

168 The scattered intensity curves were fitted using the model of sphere with attached Gaussian chain
 169 having self-avoiding walk statistics implemented in SASFit software [41] based on the model developed
 170 by Pedersen et al. [42] (Fig. 1).

171 The scattering curves in D_2O could be fitted using the following function:

$$172 \quad I(q) = P_{sgc}(q)S(q) \quad (1)$$

173 We assume that $S(q) = 1$ due to low concentration of nanoparticles in solution, 1 mg/mL. The
 174 overall scattering intensity of the sphere with attached Gaussian chain written as:

$$175 \quad P_{sgc} = N_{agg}^2\beta_{core}^2P_{core}(q) + N_{agg}\beta_{brush}^2P_{brush}(q) + 2N_{agg}^2\beta_{core}\beta_{brush}S_{brush-core}(q) +$$

$$176 \quad N_{agg}(N_{agg} - 1)\beta_{brush}^2S_{brush-brush}(q) \quad (2)$$

177 where $N_{agg}^2 \beta_{core}^2 P_{core}(q)$ is self-correlation term of the core; $N_{agg} \beta_{brush}^2 P_{brush}(q)$ is self-correlation
 178 term of the chains; $2N_{agg}^2 \beta_{core} \beta_{brush} S_{brush-core}(q)$ is the cross-term between the core and chains and
 179 $N_{agg}(N_{agg} - 1) \beta_{brush}^2 S_{brush-brush}(q)$ is the cross-term between different chains. N_{agg} is the
 180 aggregation number of polymers forming the nanoparticle per surface area, $\beta_{brush} = V_{brush}(\eta_{brush} -$
 181 $\eta_{solv})$ and $\beta_{core} = V_{core}(\eta_{core} - \eta_{solv})$ are the excess scattering lengths of a block in the corona and in
 182 the core, respectively. V_{brush} and V_{core} are the total volume of a block in the corona and in the core,
 183 respectively. η_{brush} and η_{core} are the corresponding scattering length densities (SLDs). $P_{core}(q)$ is
 184 scattering of spherical core

$$185 \quad P_{core}(q, R_{core}) = 3 \frac{(\sin(qR_{core}) - qR_{core}\cos(qR_{core}))}{(qR_{core})^3} \quad (3)$$

186 The scattering intensity for the brush is given by:

$$187 \quad P_{brush}(q, R_{gchain}) = 2 \frac{\exp(-x) - 1 + x}{x^2} \quad (4)$$

188 where $x = R_{gchain}^2 q^2$; R_{gchain} is the gyration radius of a polymer chain.

189 The contribution of cross term between core and chains which form brush of wormlike micelle is
 190 calculated using equation:

$$191 \quad S_{brush-core}(q, R_{core}, R_{gchain}, d) = \psi(qR_{gchain}) P_{core}(q, R_{core}) \frac{\sin(q[R_{core} + dR_{gchain}])}{q[R_{core} + dR_{gchain}]} \quad (5)$$

192 where $\psi(qR_{gchain}) = \frac{1 - \exp(-x)}{x}$ is the form factor amplitude of the chain.

193 The contribution of cross term between chains is calculated using equation:

$$194 \quad S_{brush-brush}(q, R_{core}, R_{gchain}, d) = \psi^2(qR_{gchain}) \left[\frac{\sin(q[R_{core} + dR_{gchain}])}{q[R_{core} + dR_{gchain}]} \right]^2 \quad (6)$$

195 where d is parameter that accounts for non-penetration of the chains into the core and should be
196 mimicked by $d \sim 1$ for $R_{core} \gg R_{gchain}$.

197 The model has the following fitting parameters: R_{core} – core radius; V_{core} – molecular volume of
198 single block unit in the micellar core; V_{brush} – molecular volume of single block unit in the micellar
199 corona; η_{core} – scattering length density of spherical core; η_{brush} – scattering length density of the block
200 unit in the corona; η_{solv} – scattering length density of solvent; R_{gchain} – gyration radius of polymer
201 chains in the corona; L – contour length of polymer chain, b – Kuhn segment length.

202 Excess scattering lengths of solvent and polymeric shell, V_{core} , V_{brush} , contour length of polymer
203 chain, Kuhn segment length were known from literature data and polymer composition and were chosen
204 to be fixed during the fitting procedure.

205 To account for nanoparticles polydispersity, a Schulz-Zimm distribution of R_{core} with
206 polydispersity parameter σ was included in the following way:

$$207 \quad SZ = \frac{R_{core}^Z}{\Gamma(Z+1)} \left(\frac{Z+1}{\langle R_{core} \rangle} \right)^{Z+1} \exp \left[-\frac{(Z+1)R_{core}}{\langle R_{core} \rangle} \right] \quad (7)$$

$$208 \quad \text{where } Z = \frac{1}{\sigma^2} - 1 \quad (8)$$

209 The gyration radius R_g of nanoparticles was calculated from Guinier regime to evaluate the overall
210 size of nanoparticles.

211 **2.7. Encapsulation efficiency and loading capacity**

212 Amicon[®] Ultra-0.5 Ultracel-3 centrifugal filter unit with a molecular weight cut-off 10 kDa was
213 used in these experiments. Each centrifugal filter device was pre-rinsed with PBS (500 μ L) at 13,000
214 rpm ($7558 \times g$) for 30 min prior to further use. The dispersion of PLGA-PEG nanoparticles (500 μ L) was
215 placed in an ultrafiltration tube and centrifuged at 4 °C at 13,000 rpm ($7558 \times g$) for 30 min. This loading

216 step was repeated twice. The filtrate was discarded and the retentate was washed with 250 μ L of PBS by
217 further centrifugation at 4 $^{\circ}$ C at 13,000 rpm ($7558 \times g$) for 20 min. The filtrate was withdrawn again and
218 the fluorescein-loaded PLGA-PEG nanoparticles in the retentate were then mixed with 100 μ L DMSO
219 (left for 6 h in a fridge to dissolve the nanoparticles and model drug) and spun at 4 $^{\circ}$ C at 13,000 rpm
220 ($7558 \times g$) for 10 min. The amount of free fluorescein in the supernatant was quantified using a Varian
221 Cary Eclipse fluorescence spectrophotometer at $\lambda_{excitation}$ and $\lambda_{emission} = 460$ and 513 nm, respectively,
222 and the encapsulation efficiency (EE%) and loading capacity (LC%) were determined using the
223 following formulae:

$$224 \quad EE\% = \frac{C}{C_i} \times 100 \quad (9)$$

$$225 \quad LC\% = \frac{C}{Total\ weight\ of\ NPs} \times 100 \quad (10)$$

226 where C is the amount of fluorescein encapsulated in the nanoparticles (NPs), and C_i is the initial amount
227 of fluorescein. A standard curve was generated by plotting the fluorescence intensities from different
228 concentrations of the model drug and used to calculate EE% and LC% can be found in Supplementary
229 Information (Fig. S1).

230 **2.8. Toxicology: slug mucosal irritation test**

231 The slug mucosal irritation test (SMIT) was carried out according to our previously published
232 report [43]. *Arion lusitanicus* slugs were collected locally in Harris Garden (Reading, UK) and were
233 housed in specially designed plastic containers and fed with lettuce, cabbage, carrots and cucumber. Each
234 slug's body lining was carefully examined and only slugs showing no evidence of macroscopic injuries
235 with clear tubercles and a foot surface were used for testing purposes. Slugs weighing between 15 and
236 23 g were isolated from the culture and were kept individually in 1.5 L glass beakers lined with a paper

237 towel soaked with 20 mL of PBS solution and left at room temperature for 48 h before the start of an
238 experiment. All beakers were covered with a cling film pierced with tiny holes in order to allow air
239 exchange. Each slug was individually weighed before the experiment and then placed in 90 mm Petri
240 dishes lined with Whatman™ filter paper moistened with either positive/negative controls (2 mL of 1%
241 BAC prepared in PBS and 2 mL of PBS solution, respectively) or 2 mL of each test materials (PEG,
242 PEG-Mal and MHA) with the following concentrations: 0.00003; 0.0003; 0.003 and 0.03 mmol prepared
243 in PBS. After 60 min contact period slugs were taken out, rinsed with 10 mL of PBS, gently wiped with
244 the paper towel and then reweighed. The percentage of mucus production (MP%) was estimated as a slug
245 body weight loss and evaluated using the following equation:

$$246 \quad MP\% = \frac{(m_b - m_a)}{m_b} \times 100 \quad (11)$$

247 where m_b and m_a are the weights of a slug before and after experiment, respectively. Each experiment
248 was repeated 7 times using different slugs and the results were evaluated statistically, calculating the
249 mean \pm standard deviation values.

250 **2.9. Preparation of artificial urine solution**

251 Artificial urine solution was prepared according to the previously reported protocol [44]. Briefly,
252 the following components were dissolved in deionised water by stirring overnight at room temperature,
253 before making the total volume to 2000 mL: urea (24.27 g), uric acid (0.34 g), creatinine (0.90 g), sodium
254 citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$, 2.97 g), sodium chloride (NaCl, 6.34 g), potassium chloride (KCl,
255 4.50 g), ammonium chloride (NH_4Cl , 1.61 g), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.89 g),
256 magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.00 g), sodium bicarbonate (NaHCO_3 , 0.34 g), sodium
257 oxalate ($\text{Na}_2\text{C}_2\text{O}_4$, 0.03 g), sodium sulphate (Na_2SO_4 , 2.58 g), sodium phosphate monobasic monohydrate

($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1.00 g), and sodium phosphate dibasic (Na_2HPO_4 , 0.11 g). The artificial urine solution (pH 6.40) was kept at 37 °C throughout the experiments.

2.10. *In vitro* retention studies on lamb urinary bladder mucosa

The retention of PLGA-PEG nanoparticles on lamb urinary bladder tissues *in vitro* was determined using a protocol previously described by our group with minor modifications [29]. Lamb bladder tissues were received from P.C. Turner Abattoirs (Farnborough, UK) immediately after animal slaughter, packed, frozen and transported in an insulated plastic container. The tissues were subsequently thawed upon arrival and carefully excised to yield approximately 2 × 3 cm sections, avoiding contact with the internal mucosa, which were then used in the experiments. The dissected bladder tissue was mounted on a glass slide with mucosal side facing upward and rinsed with 3 mL of artificial urine (AU; pH 6.40) solution. Experiments were performed with the bladder tissues maintained at 37 °C in an incubator. Aliquots (200 µL) from fluorescein-loaded PLGA-PEG nanoparticle stock solutions were withdrawn and deposited onto a mucosal surface and rinsed with AU at a constant flow rate of 2 mL/min using a syringe pump (total washing time was 50 min). Fluorescence images of a bladder tissue were taken using Leica MZ10F stereo-microscope (Leica Microsystems, UK) with Leica DFC3000G digital camera at 1.6× magnification with 30 ms exposure time (gain 2.0×), fitted with a GFP filter. The microscopy images were then analysed with ImageJ software by measuring the pixel intensity after each irrigation with AU. The pixel intensity of the blank samples (bladder mucosa without test material) were subtracted from each measurement and data are converted into numbers. All measurements were conducted in triplicate.

Evaluation of formulations retention on the mucosa *in vitro* was quantified through WO_{50} values, which represent the volume of a biological fluid necessary to wash out 50 % of a mucoadhesive excipient from a substrate [45]. WO_{50} values of test materials were calculated *via* extrapolation of the average wash-off profiles to 50% using polynomial fitting (5th order) and Wolfram Alpha (a computational knowledge engine).

2.11. Statistical analysis

Statistical analysis of data, i.e. mean values \pm standard deviations were calculated and assessed for significance using two-tailed Student's *t*-test and a one-way analysis of variance (ANOVA) followed by Bonferoni *post hoc* test using GraphPad Prism software (version 7.0), where $p < 0.05$ was fixed as the statistical significance criterion.

3. Results and discussion

3.1. Preparation and characterisation of nanoparticles

PLGA-PEG nanoparticles, with and without fluorescein, were formulated using single emulsion method and precipitated from acetone and DMSO. The detailed preparation procedure of nanoparticles is illustrated in Fig. 2. The average mean diameter of all PLGA-PEG nanoparticles precipitated from DMSO remained $\sim 100 \pm 1$ nm on average regardless of the molecular weight of PEG moiety in block copolymers, whereas the nanoparticles precipitated from acetone displayed smaller values of 65 ± 1 nm and 80 ± 1 nm for PLGA-PEG and PLGA-PEG-Mal, respectively. We believe that the difference in the particle sizes might depend on the dielectric constant of organic solvent (acetone, $\epsilon = 21$ and DMSO, $\epsilon = 47$) and their miscibility with water during formation of nanoparticles in aqueous phase [46]. It is also interesting to compare our results with the study reported by Yang et al. [47], who prepared PLGA-PEG nanoparticles from acetone, acetonitrile and tetrahydrofuran. They reported the solvent effect on the particle size with formation of 154 ± 3 , 134 ± 2 and 186 ± 4 nm of nanoparticles from acetone, acetonitrile and tetrahydrofuran, respectively. Our nanoparticles prepared from acetone are much smaller (65 ± 1 nm), which shows the strong influence of other factors, including individual block molecular weights and particle preparation techniques.

All nanoparticles prepared in the present study showed negative zeta-potential greater than -21 mV and had low polydispersity of less than 0.20, indicating the presence of a homogeneous population with

305 a narrow size distribution (Fig. 3). The physicochemical characteristics of PLGA-PEG nanoparticles are
306 summarised in Table 1.

307 Fluorescein was used as a model drug to demonstrate the potential application of PLGA-PEG
308 nanoparticles in drug delivery to urinary bladder. Fluorescein (partition coefficient, $\log P = 3.35$, [48])
309 was loaded into the nanoparticles by first preparing the model drug solution in the organic solvent
310 followed by dissolution of polymers and further nanoprecipitation in deionised water.

311 The size and morphology of nanoparticles were further confirmed by TEM analysis and
312 microphotographs are displayed in Fig. 4. Uranyl acetate was used as a negative staining to achieve
313 reasonable contrast. TEM analysis revealed the formation of homogeneous vehicles with well-dispersed
314 spherically shaped core and shell structure of nanoparticles and the results are in good agreement with
315 the data obtained by DLS measurements (Table 1).

316 **3.2. Nanoparticle structure**

317 Small-angle neutron scattering (SANS) was used to probe the nanoparticle architecture and
318 determine their molecular parameters. The use of SANS allows to get more information about small
319 particles compared to DLS method. The scattering intensity $I(q)$ curves for all nanoparticle solutions are
320 presented in Fig. 5A and 5B. Several issues should be noted from the inspection of these scattering
321 curves. First, all curves have monotonous behaviour with q ; no additional maxima are observed. It is a
322 typical manifestation of moderate or high polydispersity for compact objects. Second, all curves are
323 similar in the middle and high q ranges that implies similarity of all nanoparticles from inside
324 disregarding preparation way and used solvent. The only difference between the scattering curves is
325 visible in low q range that is probably related to the difference in the aggregation number. To have a
326 deeper information, the gyration radius values of nanoparticles in solution were calculated using Guinier
327 approximation (Table 2). Comparison of gyration and hydrodynamic radii reveals that these two
328 parameters are clearly correlated. In agreement with DLS data, the gyration radius value for PLGA-PEG

329 and PLGA-PEG-Mal nanoparticles precipitated from DMSO is nearly the same and insensitive to the
330 PEG molecular weight to the presence of maleimide groups (Table 2). In contrast, the gyration radius
331 value of nanoparticles precipitated from acetone is higher for longer length of a PEG chain. The ratio of
332 gyration and hydrodynamic radii R_g/R_h have been known as the ρ parameter that is sensitive to the
333 architecture of nano-objects. It provides model-independent clue on spatial arrangement of a scattering
334 object [49–53], its theoretical value is known for some simple models such as a hard sphere ($\rho =$
335 0.775), Gaussian chain in θ -solvent ($\rho = 1.5$), long rods ($\rho > 2.0$). The ρ parameter value calculated
336 for PLGA-PEG and PLGA-PEG-Mal nanoparticles precipitated from DMSO undoubtedly pointing out
337 on a spherical symmetry and compact structure in agreement with TEM results presented above. The
338 change from DMSO to acetone results in the formation of nanoparticles with more loose structure since
339 they have higher ρ parameter value. Increasing of PEG molecular weight leads to increase in ρ value
340 highlighting the enhanced branching structure for 5 kDa PEG nanoparticles made from acetone. We have
341 to mention here that some of these systems have ρ value that is below the lowest possible theoretical
342 limit, 0.775. Nevertheless, low values were already reported in a variety of publications. The ρ parameter
343 fluctuates around 0.8, an average value for all types of nanoparticles justifying the choice of the fitting
344 model described in Experimental Section.

345 The scattering curves obtained for the D₂O solutions can be well fitted with the “sphere with
346 attached Gaussian chain having self-avoiding walk statistics” model, assuming a Schulz-Zimm
347 distribution for core radius. The calculated structural parameters are presented in Table 2.

348 The obtained value of the core radii and shell thickness are in the range of 12–24 nm, which is in
349 agreement with the R_h as observed in the DLS measurements and TEM. In all series of nanoparticles
350 prepared by precipitation from acetone and DMSO, R_{gchain} increases with increase in PEG molecular
351 weight (Table 2), whereas R_{core} is much less sensitive to PEG chain length.

352 Our results corroborate with SANS studies of PLGA-PEG based block copolymers published
353 previously [47,54–57] keeping in mind the difference in composition, molecular weights and methods of
354 preparation. For example, PEO-PLGA-PEO [55] and PLGA-PEG-PLGA [56] triblock copolymers were
355 investigated using SANS. Apart from the difference in composition, triblock vs diblock, the copolymers
356 reported in these papers were different on other important aspects. The molecular weight, M_n , of PLGA
357 block was much lower than the one in our case, 3,500 g/mol [55] and 1,170 g/mol [56], respectively.
358 In both cases solutions were prepared by direct dissolving in D_2O in contrast with solvent exchange
359 method exploited in the present paper. Additionally, structure factor was involved in the fitting procedure
360 since the triblock copolymer solution were in semi-diluted regime, 24 and 20 wt.% for PEO-PLGA-PEO
361 and PLGA-PEG-PLGA, respectively. Applying the Percus-Yevick (PY) hard-sphere model to describe
362 the structure factor and Global Indirect Fourier Transformation (GIFT) to describe the form factor of the
363 micelles composed of PEO-PLGA-PEO at 30 °C, the authors obtained the following values for the radii
364 of a core and shell, 5.9 and 2.5 nm, respectively [55]. The presence of a shell was also reported by
365 Khorshid et al for flower-like micelles composed by PLGA-PEG-PLGA triblock copolymers [56]. SANS
366 method successfully disseminates the internal structure of nanoparticles. The SANS experiments give a
367 clear evidence of asymmetric ellipsoid nanoparticles for the copolymer with the PEG block ($M_n = 1,000$
368 g/mol), whereas spherical micelles with core-shell topology is observed for the polymer with longer
369 PEG central block ($M_n = 1,500$ g/mol). The core-shell cylinder and spherical shell models were utilised
370 to fit the SANS data [56]. Unlike the PEO-PLGA-PEO system, both PLGA-PEG-PLGA copolymers
371 were studied not only in semi-diluted but also for diluted concentration, 1 wt.%. The core and shell radii
372 for PLGA₁₁₇₀-PEG₁₅₀₀-PLGA₁₁₇₀ were 5.0 nm and these values are much lower in comparison with the
373 data reported in our manuscript, R_{core} and R_{gchain} , Table 2, that is not surprising keeping in mind much
374 higher molecular weights for PLGA and PEG of our copolymers.

375 Interesting contrast variation SANS study was performed for laponite/PEG1.0k-PLGA0.8k and
376 laponite/PEG1.0k-PLGA1.6k nanocomposites in a broad range of D_2O/H_2O mixture concentrations [57].

377 It was found that for 66 % of D₂O, the scattering from laponite is matched to the scattering of solvent.
378 The combination of core-shell spherical model in combination with Debay form factor was used for the
379 fitting the scattering profile from laponite free nanoparticles made of PEG-PLGA copolymers. The radius
380 of a core and the micelles was found 1.6 and 4.7 nm, respectively, giving the shell thickness value to be
381 of 3.1 nm [57]. We note here that the model used in our work is very close to the one reported in [57].
382 Two SANS studies that are the most relevant to our paper deal with nanoparticles composed from PLGA-
383 PEG [47] and PLA-PEG [54] diblock copolymers. In both papers, nanoparticles were prepared by solvent
384 exchange method. The spherical model with a polydisperse core of constant scattering density and
385 diffusive shell was applied for the fitting of PLA-PEG copolymers [54]. The fitting data reported for the
386 acetone as the organic solvent and copolymer with similar composition PLA₁₅₆₀₀-PEG₅₀₀₀ are in perfect
387 agreement with our results for PLGA_{20K}-PEG_{5K} (Table 2). The mean core radius of the nanoparticles
388 made of PLA₁₅₆₀₀-PEG₅₀₀₀ is 11 nm, which correlates with our results, 12 nm. The Schultz-Zimm
389 polydispersity values are almost identical, 0.27 [54] vs 0.21 in our study. The thickness of the PEG shell
390 of 6.7 nm for PLA₁₅₆₀₀-PEG₅₀₀₀ also agrees well with our data for PLGA_{20K}-PEG_{5K} that was found to be
391 6.9 nm.

392 Surprisingly, the similar systems measured recently show clearly distinctive results [47]. The
393 PLGA-PEG nanoparticles prepared by solvent exchange method presumably composed from micelles
394 that form a fractal structure inside of a nanoparticle. One of the plausible explanations could be the
395 existence of some residual reactive groups on PLGA-PEG copolymers reported in [47]. Such reactive
396 groups might form bridges between PLGA-PEG micelles.

397 The choice of the solvent used for precipitation has the greatest impact on nanoparticles structure.
398 Interestingly, nanoparticles dissolved and precipitated from acetone have not only a smaller core but also
399 lower R_{gchain} value in comparison with the NPs precipitated from DMSO. This is a rather unpredicted
400 result that might indicate a different local structure of the interface between PLGA core and PEG brush
401 and different conformation of PEG chains. Indeed, the gyration radius of a PEG chain is twice lower for

402 NPs precipitated from acetone. Such striking difference could only be explained by compaction of PEG
403 chains on the surface of nanoparticles precipitated from acetone and, in reverse, fully stretched PEG
404 conformation in case of NPs made from DMSO. This hypothesis is indirectly supported by another
405 peculiar feature observed from the SANS results fitting: the presence of maleimide terminal groups
406 increases the R_{gchain} for DMSO series, and in reverse, decreases R_{gchain} for NPs precipitated from
407 acetone (Table 2). We hypothesize that NPs prepared from DMSO have a narrow interface between
408 PLGA and PEG blocks, whereas precipitation from acetone somehow facilitates the formation of the
409 broad interface layer where PLGA and PEG coexist. Inevitably, bulky maleimide groups attached to the
410 dangling PEG chains increase the gyration radius of PEG for NPs precipitated from DMSO. For NPs
411 precipitated from acetone we might expect that some of Mal groups, due to hydrophobic nature of
412 maleimide, will have tendency to incorporate into smooth PLGA-PEG interface (see inserts in Fig. 5).
413 Provided that the nanoparticles precipitated from DMSO have the greatest presence of active maleimide
414 groups on their surface, all subsequent experiments were performed with these samples.

415 **3.3. Mucosal irritancy**

416 The information about the biocompatibility and toxicological characteristics of maleimide-
417 containing materials is currently lacking in the literature. Previously, the biocompatibility of
418 polysaccharides functionalised with maleimide groups was evaluated only by Shtenberg et al. [30] using
419 normal human dermal fibroblasts and by Sahatsapan et al. [31] using human gingival fibroblast cells.

420 The slug mucosal irritation test (SMIT) was developed by Adriaens et al. [58,59] to evaluate the
421 mucosal irritancy potential of different formulations and active ingredients. It uses terrestrial slug species,
422 which are considered to have limited sentience and so are not protected by regulations covering animal
423 experiments [58]. Normally, slugs release mucus to aid their locomotion. They also produce mucus and
424 lose body weight when in contact with irritating substrates. When their mucosal tissue is damaged the
425 slugs release additional proteins and enzymes. This indication allows for quantifiable outcomes as for

426 irritants to be classified as non-irritating, mild or severely irritating. Generally, mild irritants cause an
427 increase in mucus production, whereas severe irritants result in tissue erosion and more mucus release in
428 addition to increased production [60].

429 Previously we reported the use of SMIT for assessing mucosal irritancy of 2-
430 hydroxyethylmethacrylate and 2-hydroxyethylacrylate based random copolymers of different
431 compositions as well as blend films based on poly(acrylic acid) and methylcellulose using *Limax flavus*
432 and *Arion lusitanicus* slugs [43,61]. In the present work, we have adopted the same methodology and
433 used *Arion lusitanicus* slugs to evaluate the ability of model compounds PEG, PEG-Mal and 6-
434 maleimidohexanoic acid (MHA) to cause mucosal irritation. Fig. 6 presents the results on mucus
435 production by slugs exposed to filter paper surfaces moistened with solutions of PEG, PEG-Mal and
436 MHA of various concentrations prepared in PBS as well as positive and negative controls. In experiments
437 with 1% solution of BAC in PBS (pH 7.50), used as a positive control, slugs experienced a severe
438 discomfort, producing approximately $38 \pm 7\%$ of yellow mucus, whereas slugs exposed to PBS (used as
439 a negative control, pH 7.74) did show a low level of mucus production of $5 \pm 1\%$. These data are in good
440 agreement with our previous studies [43,61]. A significant variability of the data obtained from
441 experiments with positive control is explained by slugs' increased activity and tendency to escape a
442 contact with an irritant chemical. In all experiments with negative control and polymeric excipients slugs
443 secreted colourless mucus, which is the first demonstration of their reasonably good biocompatibility.

444 The concentration of test materials of 0.0003 mmol, used to prepare PLGA-PEG nanoparticles in
445 dispersion, was chosen as a reference for the preparation of liquid formulations with model maleimide-
446 containing compounds (MHA and PEG-Mal). Filter paper soaked with 0.03 mmol MHA (pH 4.34)
447 displayed significantly higher irritancy ($p < 0.001$) compared to PEG-Mal (0.03 mmol; MP $6 \pm 2\%$; pH
448 6.92) with the level of mucus production reaching $21 \pm 7\%$ and confirming the ability of MHA to irritate
449 mucosal epithelia due to, mainly, its acidic nature (Fig. 6). Additionally, slugs exposed to positive control
450 exhibited an extreme discomfort ($p < 0.0001$) compared to slugs in response to contact with 0.0003 mmol

451 PEG-Mal. However, filter paper soaked with increasing concentrations of PEG and PEG-Mal
452 demonstrated low irritation potential as no significant differences ($p > 0.5$) were observed between these
453 materials and the negative control. Fig. S2 and Fig. S3 in Supplementary Information provide the detailed
454 schematic illustration of SMIT test and the photographs of mucus production by *Arion lusitanicus* slugs
455 exposed to various test chemicals, respectively. Safety, non-toxicity, and biocompatibility of PEG-Mal
456 allows us to conclude that PLGA-PEG-Mal nanoparticles should not cause irritation in the bladder lining.
457 The non-irritant nature of maleimide-functionalised materials established in this study is also in good
458 agreement with the data reported by Shtenberg et al. [30], who reported that alginate modified with
459 maleimide-terminated PEG is non-toxic to normal human dermal fibroblasts. The maleimide-
460 functionalised chitosan was also reported by Sahatsapan et al. [31] to be non-toxic to gingival fibroblast
461 cells (HGF) up to 1000 $\mu\text{g/mL}$ of polymer in solution.

462 **3.4. Mucoadhesion studies**

463 The retention properties of fluorescein-loaded PLGA-PEG and PLGA-PEG-Mal nanoparticles on
464 lamb urinary bladder mucosa were evaluated using a flow-through method with fluorescent detection
465 using the methodology described in our previously published paper [29]. Fig. 7 shows exemplary
466 fluorescent images of the retention of PLGA-PEG and PLGA-PEG-Mal nanoparticle dispersions on
467 urinary bladder mucosa irrigated with AU. After analysis of the fluorescent images using ImageJ
468 software, it was established that maleimide-functionalised PLGA_{20K}-PEG_{5K}-Mal and PLGA_{20K}-PEG_{3K}-
469 Mal nanoparticles exhibited greater mucoadhesive properties compared to unmodified counterparts ($p <$
470 0.001), confirming that PLGA-PEG-Mal nanoparticles adhere well to the bladder mucosa by forming
471 covalent linkages with thiol groups present in mucin layer of the bladder epithelium (Fig. 8). Such
472 selective binding leads to increased urothelium cell uptake and potentially improved drug bioavailability
473 within the bladder wall. The nanoparticles remained on the bladder mucosa even after 50 min of washing
474 with a cumulative AU volume of 100 mL. This is in good agreement with our previous findings [28,29],

475 therefore the results of retention study confirm the mucoadhesive properties of maleimide-functionalised
476 PLGA-PEG nanoparticles, which could also be used as potential mucoadhesive drug carriers in IDD to
477 urinary bladder.

478 Previously we have introduced a novel quantitative method of evaluating and comparing the
479 retention efficacy of liquid formulations on mucosal membranes through the use of WO_{50} values, which
480 represent the volume of a biological fluid necessary to wash out 50 % of the mucoadhesive test material
481 from a substrate [45]. In this study, we employed the same method and calculated WO_{50} values by
482 analysing individual wash-off profiles for each nanoparticle suspensions and the results are presented in
483 Table 1. By comparing these values for different PLGA-PEG nanoparticles used in this study, it is clear
484 that PLGA_{20K}-PEG_{5K}-Mal have greater retention on lamb bladder mucosa ($WO_{50} = 15$ mL, $R^2 = 1$)
485 compared to unmodified PLGA_{20K}-PEG_{5K} ($WO_{50} = 5$ mL, $R^2 = 0.9872$).

486 **4. Conclusions**

487 Four different types of PLGA-PEG nanoparticles were prepared and evaluated in this study for
488 their physicochemical characteristics and retention on urinary bladder mucosa. The nanoparticles
489 decorated with maleimide functional groups demonstrated greater retention on the bladder mucosa *in*
490 *vitro* due to their ability to form covalent linkages with thiol groups of glycoproteins expressed on the
491 bladder epithelial membrane. The biocompatibility of PEG-Mal was confirmed by SMIT assay
492 experiments.

493 The development of mucoadhesive drug delivery systems that could improve drug residence in the
494 bladder will clearly be beneficial for the treatment of bladder cancer. However, all existing strategies to
495 develop mucoadhesive materials do not provide dosage forms that could selectively bind to cancer cells
496 without adhesion to healthy epithelial cells. The development of systems that could target cancer cells in
497 the bladder is one of the challenges for future research and development in this area.

498 The maleimide-functionalised PLGA-PEG mucoadhesive nanoparticles developed in this work
499 could potentially be considered as a platform technology that could be used for drug delivery not only to
500 the bladder but also to other mucosal routes of drug administration. Further research will be focused on
501 biodegradation of these nanoparticles and studies of anticancer drugs encapsulation and release.

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510 **Conflict of interest**

511 The authors have no conflicts of interest to disclose.

512 **Author contributions**

513 The manuscript was written through contributions of all authors. All authors have given approval
514 to the final version of the manuscript.

515 **Appendix A. Supplementary material**

516 Supplementary data associated with this article is available free of charge. It contains a standard
517 curve used to determine the amount of encapsulated fluorescein in the PLGA-PEG nanoparticles (Fig.
518 S1); detailed schematic illustration of SMIT test (Fig. S2) and the photographs of mucus production by
519 *Arion lusitanicus* slugs in contact with test materials (Fig. S3).

520 **Abbreviations**

521 AU, artificial urine; BAC, benzalkonium chloride; BC, bladder cancer; D₂O, deuterium oxide;
522 DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; EE%, encapsulation efficiency; IDD,
523 intravesical drug delivery; LC%, loading capacity; Mal, maleimide; MHA, 6-Maleimidohexanoic acid;
524 MP%, mucus production; NPs, nanoparticles; PBS, phosphate buffered saline; PDI, polydispersity index;
525 PEG, poly(ethylene glycol) methyl ether; PEG-Mal, methoxypolyethylene glycol maleimide; PLGA-
526 PEG, poly(lactide-co-glycolide)-block-polyethylene glycol); SANS, small-angle neutron scattering;
527 SMIT, slug mucosal irritation test; TEM, transmission electron microscopy; UA, uranyl acetate; WO₅₀,
528 Wash Out 50%.

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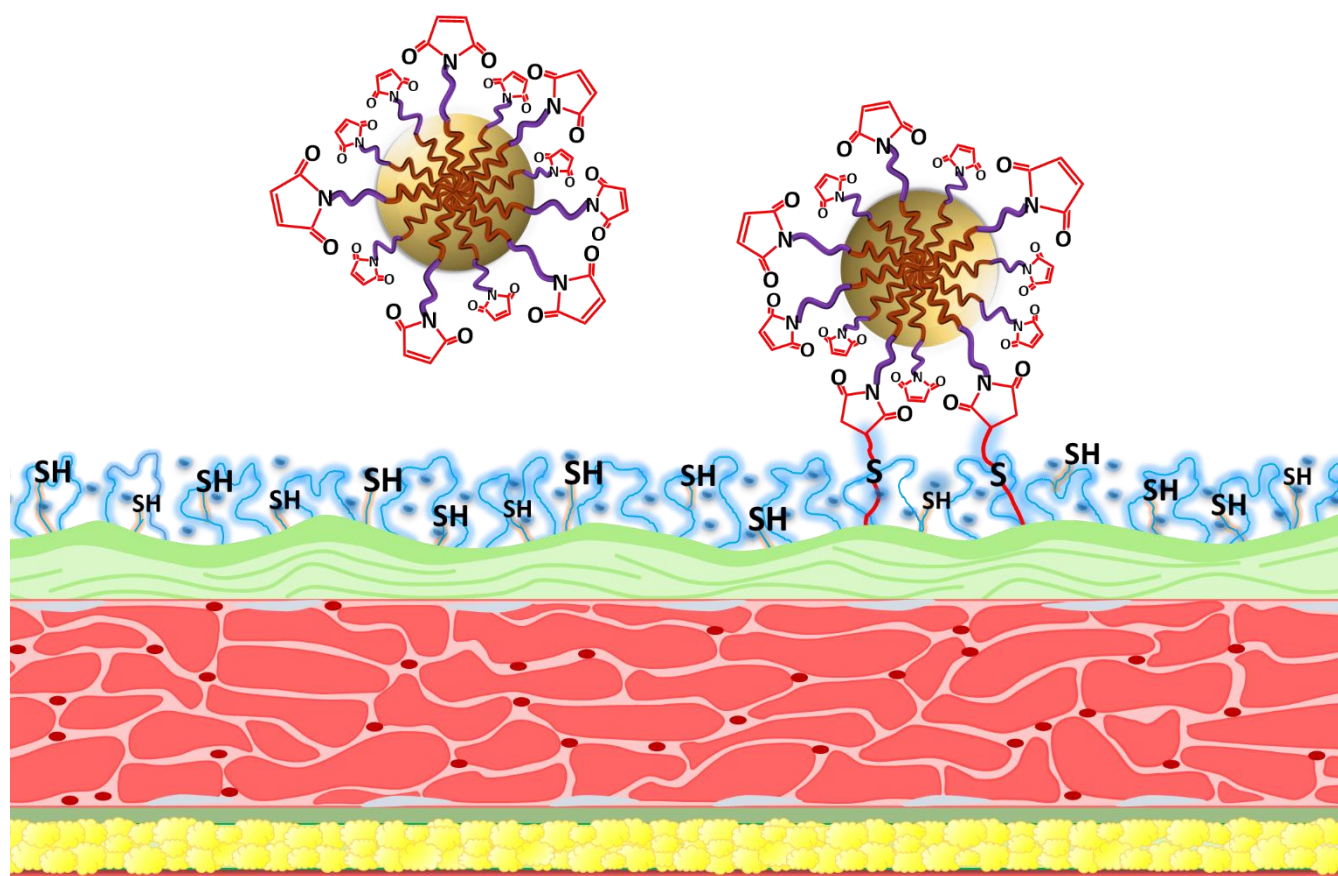
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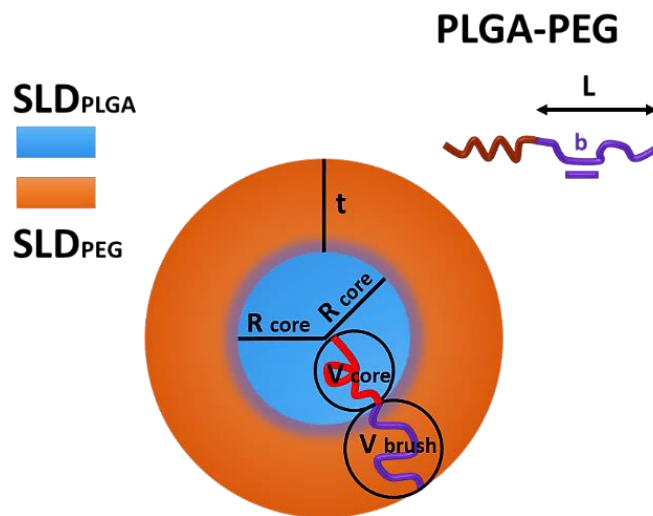


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715 Schematic illustration depicting the mechanism of enhanced mucoadhesion of PLGA-PEG-Mal
716 nanoparticles on urinary bladder mucosa.

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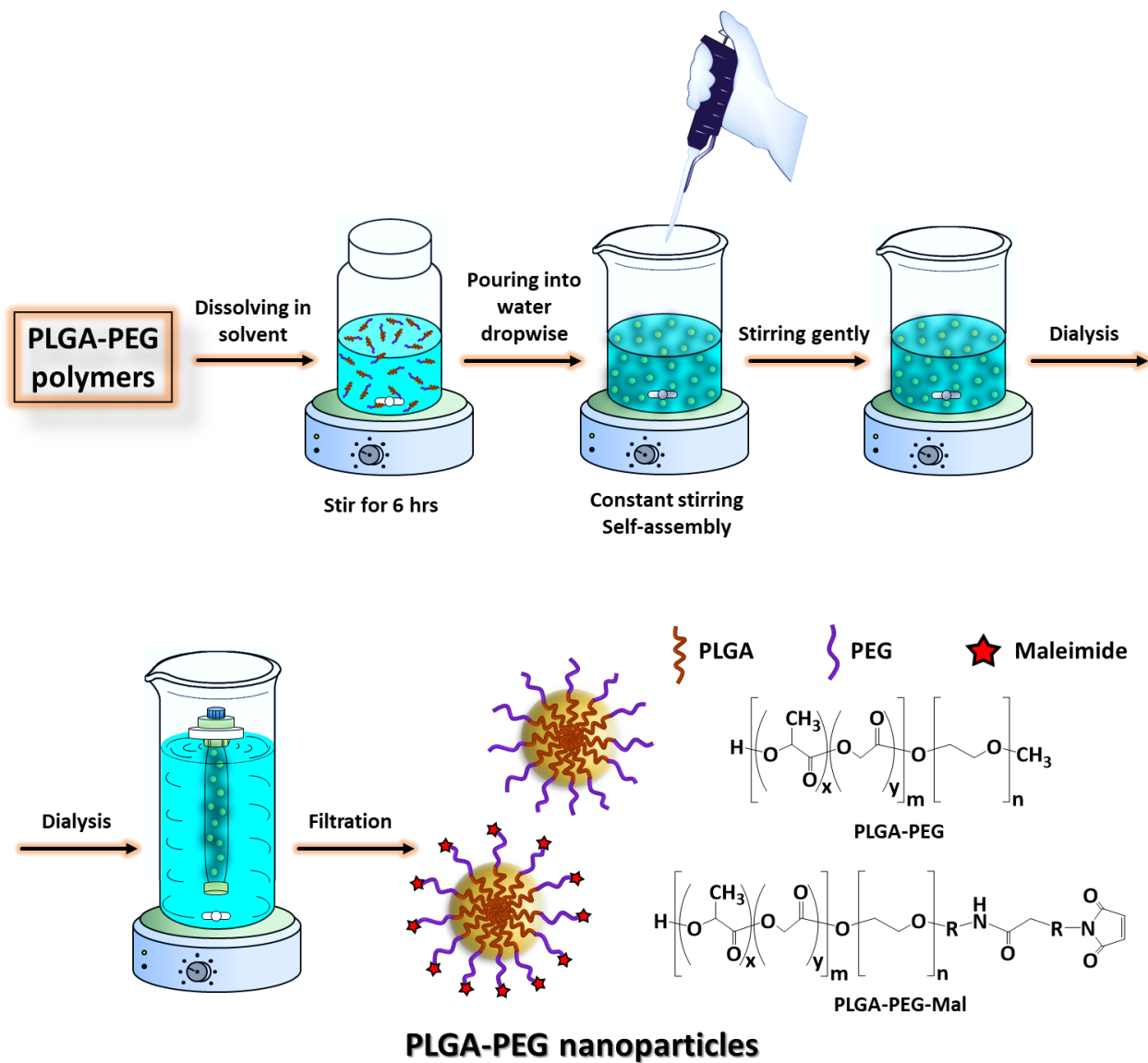
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721 **Fig. 1.** The model used for the fitting of experimental SANS data.

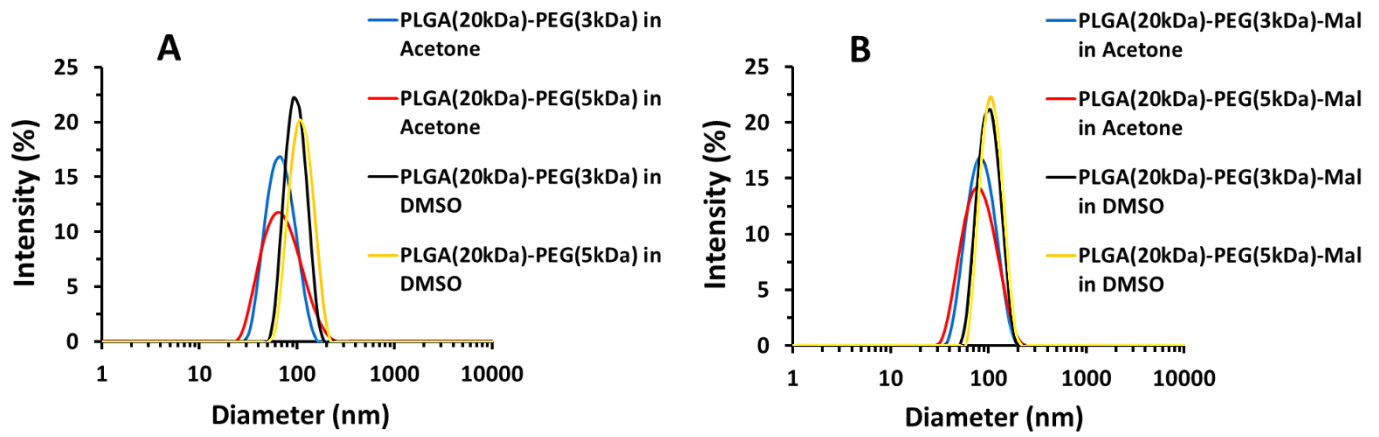
722 (single column fitting image)



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724 **Fig. 2.** Illustrative diagram depicting the preparation of PLGA-PEG nanoparticles with maleimide-
 725 functionalised surface.

726 (2-column fitting image)



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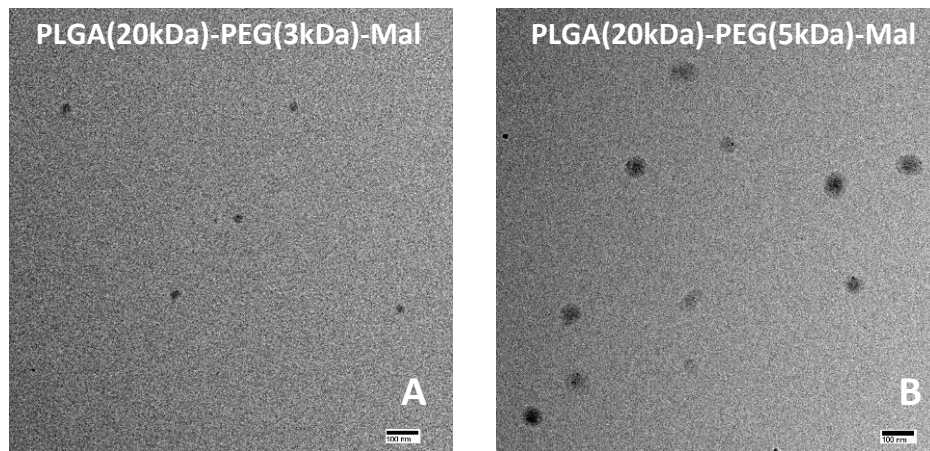
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Fig. 3. Size distribution of PLGA-PEG (A) and PLGA-PEG-Mal (B) nanoparticles dissolved and precipitated from acetone and DMSO as determined by DLS.

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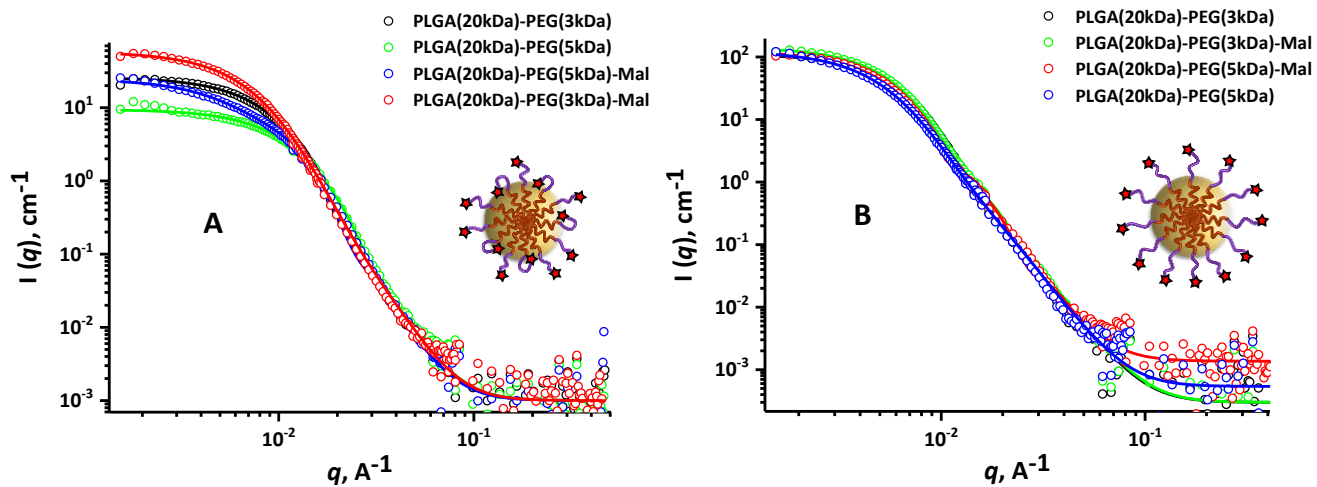
(2-column fitting image)



731

732 **Fig. 4.** TEM microphotographs of PLGA-PEG-Mal NPs dissolved and precipitated from acetone (A) and
733 DMSO (B). Scale bars are 100 nm.

734 (single column fitting image)

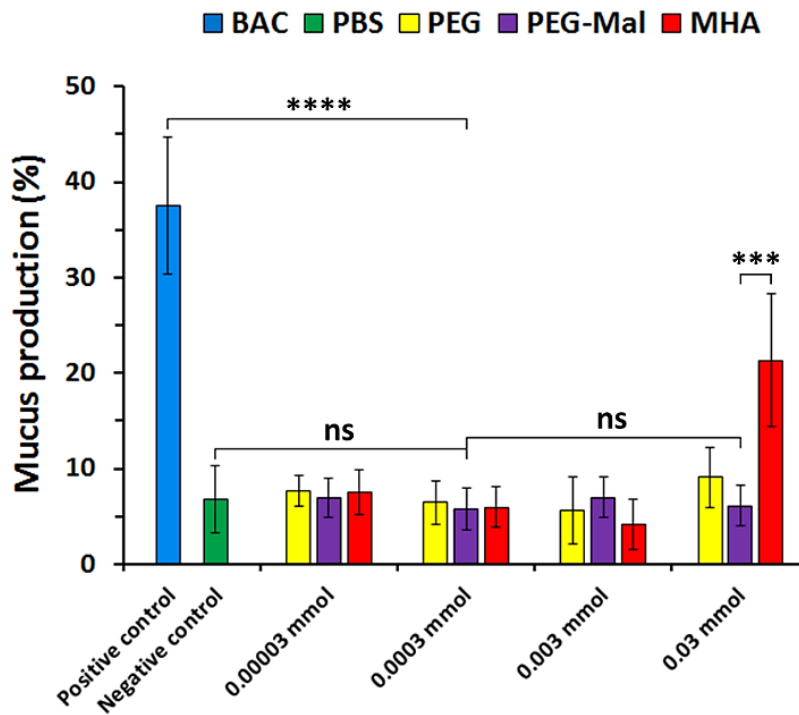


735

736 **Fig. 5.** SANS curves for PLGA-PEG nanoparticles dissolved and precipitated from acetone (A) and

737 DMSO (B). Solid line is a fitting curve. Inserts: Proposed structures of nanoparticles.

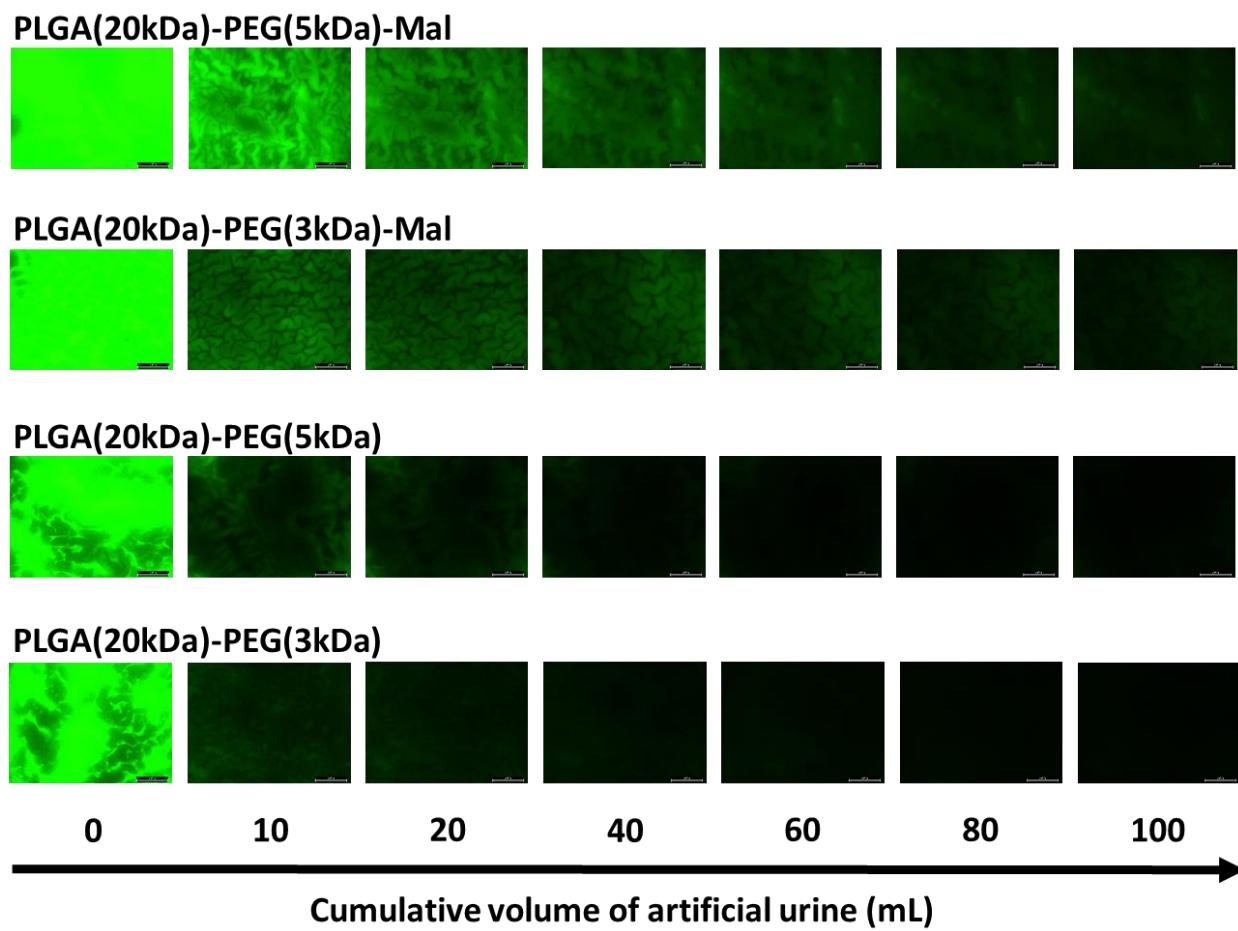
738 (2-column fitting image)



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740 **Fig. 6.** Mucus production by *Arion lusitanicus* slugs in response to 60 min exposure to PEG, PEG-Mal
 741 and MHA as well as positive (benzalkonium chloride – BAC) and negative (phosphate buffered saline –
 742 PBS) controls. Data are expressed as mean \pm standard deviation (n = 7). Statistically significant
 743 differences are given as: **** – $p < 0.0001$; *** – $p < 0.001$; ns – no significance.

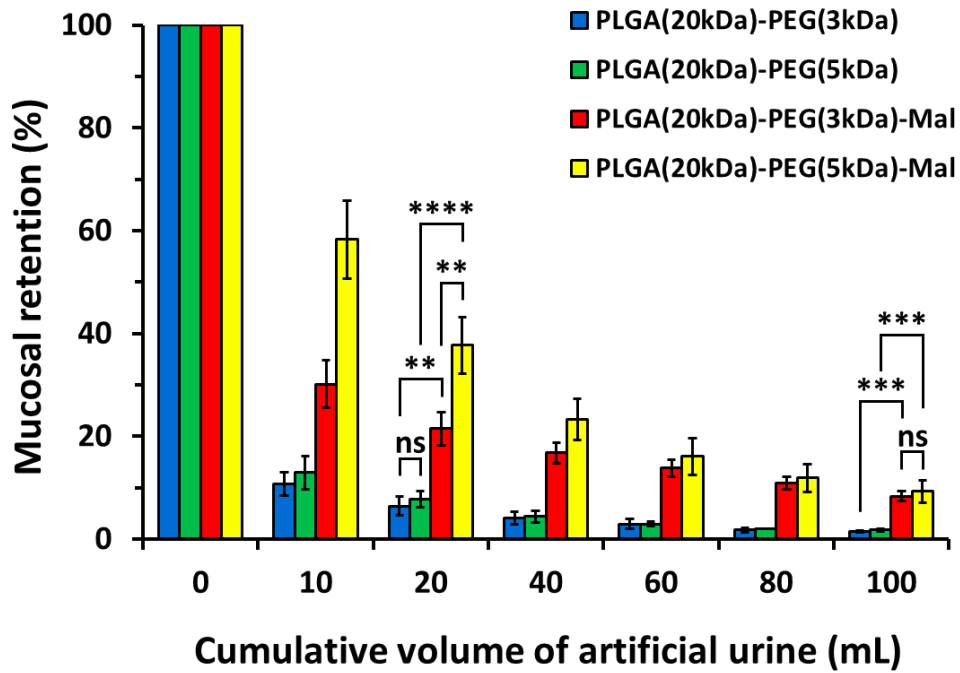
744 (single column fitting image)



745

746 **Fig. 7.** Exemplar fluorescence images showing retention of PLGA-PEG nanoparticles on lamb urinary
 747 bladder mucosa washed with different volumes of AU. Scale bars are 2 mm.

748 (2-column fitting image)



749

750 **Fig. 8.** Percentage retention of PLGA-PEG nanoparticles on lamb urinary bladder mucosa after irrigating
 751 with different volumes of AU solution. Data are expressed as mean \pm standard deviation (n = 3).
 752 Statistically significant differences are given as: **** – $p < 0.0001$; *** – $p < 0.001$; ** – $p < 0.01$; ns
 753 – no significance.

754 (single column fitting image)

755

TABLES WITH CAPTIONS

756

Table 1. Physicochemical characteristics of PLGA-PEG nanoparticles.

Formulation	Solvent used in NPs preparation	Mean diameter (nm)	PDI	Zeta- potential (mV)	EE%	LC%	WO ₅₀ (mL)*
PLGA _{20K} -PEG _{3K}	DMSO	94 ± 1	0.048	-20.8 ± 0.5	62.7 ± 3.6	3.1 ± 0.2	4; (R ² = 0.9860)
PLGA _{20K} -PEG _{5K}		105 ± 1	0.070	-21.6 ± 0.4	63.8 ± 6.9	3.2 ± 0.4	5; (R ² = 0.9872)
PLGA _{20K} -PEG _{3K} -Mal		98 ± 1	0.060	-17.7 ± 0.6	60.0 ± 4.2	3.0 ± 0.2	6; (R ² = 0.9931)
PLGA _{20K} -PEG _{5K} -Mal		104 ± 1	0.067	-12.2 ± 0.5	55.0 ± 5.8	3.0 ± 0.3	15; (R ² = 1)
PLGA _{20K} -PEG _{3K}	Acetone	64 ± 1	0.110	-8.1 ± 0.7	N/A	N/A	N/A
PLGA _{20K} -PEG _{5K}		68 ± 1	0.248	-5.2 ± 0.6	N/A	N/A	N/A
PLGA _{20K} -PEG _{3K} -Mal		80 ± 1	0.094	-16.5 ± 0.6	N/A	N/A	N/A
PLGA _{20K} -PEG _{5K} -Mal		81 ± 1	0.206	-10.8 ± 0.8	N/A	N/A	N/A

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DMSO, dimethyl sulfoxide; PLGA-PEG, poly(lactide-*co*-glycolide)-*block*-polyethylene glycol); Mal, maleimide; PDI, polydispersity

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index; EE%, encapsulation efficiency; LC%, loading capacity; WO₅₀, Wash Out 50% profile is a volume of artificial urine required to

759

wash out 50% liquid formulation; N/A, not applicable. *Polynomial fitting (5th order) was used to quantify WO₅₀ values. Results are

760

given as mean ± standard deviation (n =3).

761

(2-column fitting table)

762

Table 2. Comparison of structural parameters obtained from samples PLGA-PEG nanoparticles in D₂O

Formulation	Solvent used in NPs preparation	R_g (nm)	R_g/R_h	R_{core} (nm)	σ	R_{gchain} (nm)
PLGA _{20K} -PEG _{3K}	DMSO	35.7 ± 1.4	0.76	14.8 ± 0.1	0.43	12.0 ± 0.1
PLGA _{20K} -PEG _{5K}		40.8 ± 1.5	0.78	15.4 ± 0.2	0.63	12.1 ± 0.3
PLGA _{20K} -PEG _{3K} -Mal		35.7 ± 0.9	0.73	14.6 ± 0.1	0.31	14.4 ± 0.2
PLGA _{20K} -PEG _{5K} -Mal		35.5 ± 0.7	0.68	15.8 ± 0.2	0.20	16.7 ± 0.3
PLGA _{20K} -PEG _{3K}	Acetone	21.0 ± 0.8	0.66	10.2 ± 0.2	0.33	6.9 ± 0.1
PLGA _{20K} -PEG _{5K}		36.0 ± 0.5	1.06	12.0 ± 0.2	0.21	8.6 ± 0.1
PLGA _{20K} -PEG _{3K} -Mal		30.0 ± 1.0	0.75	12.8 ± 0.1	0.50	4.1 ± 0.2
PLGA _{20K} -PEG _{5K} -Mal		33.0 ± 2.0	0.81	12.0 ± 0.1	0.24	8.3 ± 0.1

763

DMSO, dimethyl sulfoxide; PLGA-PEG, poly(lactide-*co*-glycolide)-*block*-polyethylene glycol); Mal, maleimide; R_g , overall gyration

764

radius of a nanoparticle; R_{core} , core radius; σ , polydispersity parameter; R_{gchain} , gyration radius of a polymer chain in the corona.

765

(single column fitting table)