

# **Non-Covalent Hydrogels of Cyclodextrins and Poloxamines for the Controlled Release of Proteins**

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1 **ABSTRACT:** Different types of gels were prepared by combining poloxamines (Tetronic), i.e.  
2 poly(ethylene oxide)/poly(propylene oxide) (PEO/PPO) octablock star copolymers, and  
3 cyclodextrins (CD). Two different poloxamines with the same molecular weight (ca. 7000) but  
4 different molecular architectures were used. For each of their four diblock arms, direct Tetronic  
5 904 presents PEO outer blocks while in reverse Tetronic 90R4 the hydrophilic PEO blocks are  
6 the inner ones. These gels were prepared by combining  $\alpha$ -CD and poloxamine aqueous solutions.  
7 The physicochemical properties of these systems depend on several factors such as the structure  
8 of the block copolymers and the Tetronic/ $\alpha$ -CD ratio. These gels were characterized using  
9 differential scanning calorimetry (DSC), viscometry and X-ray diffraction measurements. The  
10 90R4 gels present a consistency that makes them suitable for sustained drug delivery. The  
11 resulting gels were easily eroded: these complexes were dismantled when placed in a large  
12 amount of water, so controlled release of entrapped large molecules such as proteins (Bovine  
13 Serum Albumin, BSA) is feasible and can be tuned by varying the copolymer/CD ratio.

14

## 15 **Highlights**

- 16 • Direct and reverse poloxamines yield supramolecular gels when interacting with  $\alpha$ -  
17 cyclodextrin in aqueous media, but solid precipitates are obtained in the case of  $\beta$ -  
18 cyclodextrin.
- 19 • The rheological behaviour of the poloxamine/ $\alpha$ -cyclodextrin gels depend on the  
20 copolymer/ oligosaccharide ratio and the temperature.
- 21 • Proteins or other molecules can be loaded into these supramolecular structures before  
22 gelation.
- 23 • The resulting gels were easily eroded so controlled release of entrapped proteins is  
24 feasible and can be tuned by varying the copolymer/CD ratio.

25

26 **Keywords**

27 Cyclodextrins, self-assembly, host-guest interactions, controlled release

28

29 **INTRODUCTION**

30 Hydrogels are crosslinked polymeric networks with a soft and hydrophilic nature that makes  
31 them suitable in numerous biomedical applications such as matrices for drug delivery, wound  
32 healing, or tissue engineering (Ko et al., 2013). The self assembled hydrogels, i.e. those  
33 possessing a physical crosslinking, have attracted much attention lately. These hydrogels can be  
34 useful in pharmaceutical and biological applications, especially for some purposes as gene  
35 carriers or in the delivery of delicate bioactive agents such as drugs or proteins (Koopmans and  
36 Ritter, 2008, Li et al., 2006, Li and Loh, 2008, Lin et al., 2013, Ni et al., 2009, Nielsen et al.,  
37 2009, van de Manakker et al., 2008, Van Tomme et al., 2008, Wintgens et al., 2008). Among  
38 their promising properties, they present a quick degradation process and the capability of forming  
39 the gel “in situ” by physical crosslinking between molecules coming from different solutions. In  
40 this process of gelation, a protein, a peptide, or a drug can be added to the mixture so it will  
41 finally become immobilized inside the bulk of the gel (Gref et al., 2006, Li et al., 2006). This  
42 particular property is very significant because it allows us to inject the mixture inside a tissue  
43 using a simple needle without any kind of surgery (Li et al., 2003a).

44       Amphiphilic molecules such as polyethylene oxide/polypropylene oxide (PEO/PPO)  
45 block copolymers exhibit interesting self-assembling properties. The behaviour of amphiphilic  
46 triblock PEO-PPO-PEO copolymers (poloxamers or Pluronics) can be tailored by modifying the  
47 molecular weight and the PEO/PPO ratio within the copolymer (Alexandridis and Hatton, 1995,  
48 Trong et al., 2008). If the distribution of the hydrophilic and hydrophobic blocks is inverted, the

49 resulting PPO-PEO-PPO poloxamers (Pluronic R or reverse) show a different behaviour in water  
50 solutions (Zhou and Chu, 1994).

51 Tetronics or poloxamines are four armed copolymers that contain two blocks per arm, a  
52 PEO block and a PPO block. Normal or direct Tetronics present outer PEO blocks, while reverse  
53 Tetronics posses inner PEO blocks. The behaviour of normal Tetronics in water has already been  
54 reported (Na et al., 1999) although only a couple of works deal with reverse Tetronics (Plestil et  
55 al., 2003, Larrañeta and Isasi, 2013). Some Tetronics can form aggregates such as micelles and,  
56 in some instances, they can gel under certain conditions, so poloxamines display a great potential  
57 as components of drug delivery systems and tissue engineering devices (Alvarez-Lorenzo et al.,  
58 2007, Gonzalez-Lopez et al., 2008).

59 In previous works, some authors have discovered that mixing PEO/PPO block copolymers  
60 with cyclodextrins (CD) produces self-assembled gels (Koopmans and Ritter, 2008, Li et al.,  
61 2006, Nielsen et al., 2009, Tan et al., 2012). Cyclodextrins are natural molecules derived from  
62 starch with a relatively hydrophobic cavity, which can form inclusion complexes with different  
63 molecules, including linear polymers (Dodziuk, 2006). In this case, the resulting complex is  
64 called a polypseudorotaxane (Li et al., 2003a, Li et al., 2006, Ni et al., 2009). For instance,  $\alpha$ -CD  
65 molecules yield inclusion complexes with PEO and its block copolymers (Harada et al., 1995,  
66 Harada, 1998, Harada et al., 2009, Harada, 1997, Li et al., 2001). On the other hand,  
67 cyclodextrins with a wider rim (i.e.  $\beta$ - and  $\gamma$ -CD) are capable of forming complexes with PPO  
68 blocks.

69 Several works regarding the formation of inclusion complexes and gels between  
70 poloxamers (Pluronic) and CDs have been published (Li et al., 2003b, Li and Loh, 2008, Ni et  
71 al., 2009). The complexes between normal poloxamines (Tetronics) and CDs have been recently  
72 studied (Simões et al. 2013). In this work, we have used two octablock PEO/PPO copolymers:  
73 Tetronic 904 and its corresponding reverse version, Tetronic 90R4. These two medium sized

74 copolymers have practically the same block lengths (16-18 units), but a different architecture.  
75 Our main purpose is to ascertain whether the properties of the obtained gels depend on these  
76 architectures. After their physical characterization, the gels were tested as controlled release  
77 matrices by evaluating their erosion in water media and the release kinetics of a model protein,  
78 Bovine Serum Albumin (BSA).

79

## 80 **EXPERIMENTAL SECTION**

81 **Materials.** Tetronic 90R4 is a viscous yellow liquid, whose molecular weight given by the  
82 manufacturer is  $M_{90R4} = 6900$  g/mol. Its chemical composition was determined by  $^1\text{H-NMR}$   
83 (Bruker DPX 300):  $\text{PO}_{16}\text{EO}_{18}$  per arm (see Figure A1, Supplementary data). Tetronic 904 is a  
84 colorless viscous paste with a molecular weight of  $M_{90R4} = 6700$  g/mol. The composition of the  
85 blocks was also determined by  $^1\text{H-NMR}$ :  $\text{PO}_{15}\text{EO}_{19}$  per arm. According to their safety data sheets  
86 (BASF), these products are non-irritant to the skin, practically nontoxic for dermal applications,  
87 and show a low oral toxicity ( $\text{LD}_{50} > 5,000$  mg/kg). Both cyclodextrins,  $\alpha$ -CD and  $\beta$ -CD, were  
88 obtained from Wacker Chemie AG and were used without further purifications. Bovine Serum  
89 Albumin (BSA) was purchased from Sigma-Aldrich and used as received.

90 **Preparation of complexes and gels.** All complexes were formed mixing a determinate amount  
91 of Tetronic with an aqueous solution of CD followed by vigorous stirring. In the case of  
92 poloxamine 904, the copolymer paste was dissolved in a certain amount of water prior to its  
93 mixing with the CD solutions, in order to facilitate the homogenization process. Before any  
94 measurements, the resulting mixtures were kept at room temperature for at least one night. In the  
95 case of the  $\alpha$ -CD mixtures, the viscous fluids were used as obtained, without further purifications.  
96 On the other hand, for  $\beta$ -CD complexes, a white powder appears at the bottom of the flask when  
97 the two solutions are mixed. The supernatant was carefully removed and the powder precipitate

98 was freeze-dried and washed with tetrahydrofuran (THF) in order to remove the excess of  
99 uncomplexed poloxamine (Harada, 1998).

100 **Phase diagrams.** A series of Tetronic/ $\alpha$ -CD/water gel samples were prepared in 8 mL vials  
101 varying the  $\alpha$ -CD/copolymer ratio. The samples were placed in a water bath equipped with a  
102 thermostatic head. The state of the samples was evaluated for each temperature by direct  
103 observation. It was characterized as “gel” or “sol” according to its fluidness when the flask was  
104 kept inverted during at least 10 s. Cylindrical 23 mm diameter glass vials containing 5 g samples  
105 were used for this analysis. The influence of experimental conditions on the results of this  
106 experimental procedure was discussed in a previous work (Larrañeta and Isasi, 2012). The  
107 temperature ranged between 10 °C and 60 °C ( $\pm 0.1$  °C), using 5 °C steps; a stabilization time of  
108 10 minutes was considered as an appropriate delay time prior to testing the mixtures at each  
109 temperature.

110 **Wide-angle X-ray diffraction.** The diffractograms of  $\beta$ -CD complexes and  $\alpha$ -CD gels were  
111 carried out in a Brüker D8 Advance X ray diffractometer equipped with a X ray generator,  
112 Kristalloflex K760, using the radiation  $K_{\alpha 1}$  of the Cu ( $\lambda=1.5417$  Å), and a scanning speed of 0.4 °  
113 per minute. The  $\alpha$ -CD/Tetronic hydrogels were vacuum dried at 60 °C overnight prior to the  
114 measurements.

115 **Viscosity measurements.** The viscosity of the  $\alpha$ -CD/copolymer hydrogels was evaluated using a  
116 Haake Viscotester 550 rotational viscometer equipped with a thermostatic bath Thermo Phoenix  
117 II. Two rotors were used: the SV rotor was used for all 90R4/CD mixtures and for those 904/CD  
118 mixtures with higher viscosities, and the NV was used for 904/CD mixtures with a low viscosity  
119 (gels with 10% and 15% of the poloxamine). All measurements were performed at 25 °C and the  
120 range of shear rates covered was between 5 and 40 s<sup>-1</sup>. After a stabilization time of 30 seconds, an  
121 average of 100 data points was recorded during a measuring period of 60 seconds.

122 **DSC analyses.** The thermal analysis of the gels was performed using a DSC Mettler TA4000.  
123 The samples were kept at 140 °C for 20 min to remove the excess water. Two scans were  
124 registered between -100 °C and 140 °C using a scan speed of 20 °C/min. Glass transition  
125 temperatures were calculated as the half-height of the corresponding heat-capacity jump. All  
126 reported values were determined in the second DSC run.

127 ***In vitro* release and erosion kinetics.** The release studies were performed from 500 mL of pH 7  
128 phosphate buffer solutions using a SOTAX AT 7 Smart USP dissolution testing device at 37 °C  
129 and 25 rpm stirring speed. The gels (20 g) were formed at the bottom of the device vessels by  
130 mixing 14.3 g of a 14%  $\alpha$ -CD solution (including 150 mg of BSA) and the appropriate amounts  
131 of poloxamine and water needed to reach the required gel compositions. Then, the dissolution  
132 medium was carefully added to the top at the beginning of the release kinetics. Aliquots (5 mL)  
133 were withdrawn according to a sampling time program of about 10 h, and these sample volumes  
134 were replaced with fresh medium. The BSA concentration in each sample was evaluated both by  
135 UV-vis (Hewlett Packard 8452A) and fluorescence spectrometries (Perkin Elmer LS 50 B). Both  
136 the poloxamine and the substrate (BSA) absorb in the same UV region; in addition, the  
137 fluorescence of BSA is quenched by the poloxamine that is also released as the matrices are  
138 eroded. Therefore, a combination of the results obtained from these two techniques had to be  
139 used in order to determine the amount of substrate released as a function of time (see  
140 Supplementary data).  $\alpha$ -CD, which is also present in the solution during the erosion process, is  
141 not a quencher of BSA. The amount of  $\alpha$ -CD released from the matrix was evaluated using size  
142 exclusion chromatography (SEC) (Waters 600E system equipped with a Waters 2414 Refractive  
143 Index detector and an Aquagel OH-30 column). The erosion kinetic curves were constructed  
144 evaluating the amount of  $\alpha$ -CD dissolved in the samples as a function of time. Acidic and basic  
145 media were prepared using HCl 0.1 M or NaOH 0.1 M, respectively.

146

## 147 RESULTS AND DISCUSSION

148 **Complexation between cyclodextrins and 90R4 or 904.** When 90R4 or 904 poloxamine  
149 aqueous solutions are mixed with  $\alpha$ -CD solutions in certain ratios, a viscous white hydrogel is  
150 formed. The evidence of complexation between EO blocks (either in PEO homopolymer or in its  
151 copolymers) and  $\alpha$ -CD molecules is a well known fact (Larrañeta and Isasi, 2012, Li et al.,  
152 2003b, Ni et al., 2009). Both the complexed EO units and the uncomplexed PO blocks tend to  
153 self-associate, the first ones by interactions between the CD moieties and the second ones by  
154 hydrophobic interactions (see Figure 1). Hydrophobic associations seem to be more difficult in  
155 the case of Tetronic 904 once PEO are threaded due to steric hindrance. On the other hand,  
156 associations between PPO blocks threaded by  $\alpha$ -CD would be possible in both instances. By  
157 varying the  $\alpha$ -CD/copolymer ratio and the amount of water, two different types of aggregates can  
158 be obtained: either white viscous fluids or gels that remain stable when the flask is inverted. In  
159 addition, it has to be pointed out that the formation process is considerably faster for the normal  
160 poloxamine (904) than for the reverse one (90R4), a result attributed to the disposition of the  
161 PEO blocks in the copolymers. For the reverse poloxamine, the PEO blocks are located in the  
162 inner part of the chains so  $\alpha$ -CD molecules must surpass the PPO blocks in order to reach the  
163 PEO blocks and form the resulting polyrotaxane type structures, as occurs for PPO-PEP-PPO  
164 copolymers (Li et al., 2003b). In the case of 904 star octablock copolymers, the PEO blocks are  
165 in the outer part of the chains so the kinetics of complexation are faster. For 904 the process  
166 takes place in a few seconds, while in the case of 90R4 complexes, they are formed within 20  
167 minutes.

168 Figures 2 and 3 show the sol-gel phase diagrams for the systems 90R4/ $\alpha$ -CD and 904/ $\alpha$ -  
169 CD, respectively (the solubility limit for  $\alpha$ -CD aqueous solution is ca. 14 wt %). Tetronic 90R4  
170 can not gel by itself in water solutions (Larrañeta and Isasi, 2013, Plestil et al., 2003) in contrast  
171 to Tetronic 904, which forms gels under certain conditions of concentration and temperature



172 (Alvarez-Lorenzo et al., 2007) (above 40% wt., see Figure A2, Supplementary data). As can be  
173 seen in the phase diagrams, at least 5% (w/w) of  $\alpha$ -CD is needed to produce gels for both normal  
174 and reverse poloxamines. Interestingly, this threshold value matches the one found for a  
175 considerably larger normal poloxamine, Tetronic 908, with PO<sub>21</sub>EO<sub>114</sub> per arm (Simões et al.,  
176 2013). It is noticeable that, for intermediate  $\alpha$ -CD concentrations (5%-7%), the gel zone for the  
177 reverse Tetronic mixtures corresponds to higher 90R4/ $\alpha$ -CD ratios. In contrast, for the direct  
178 Tetronic (Figure 3), the gel zone appears at the left hand side of the diagrams, i.e. for lower  
179 904/ $\alpha$ -CD ratios. As can be seen in Figure 1, the outer PEO blocks can be threaded with higher  
180 amounts of cyclodextrin with a low steric hindrance. Then, the interaction between the resulting  
181 rotaxane structures is easily achieved through the favourable interactions between CD molecules.  
182 For the mixture between 10% of  $\alpha$ -CD with Tetronic 904, the phase diagram shows a second gel  
183 region at the right hand side of the phase diagram. An additional mechanism for gel formation  
184 comes into play: the hydrophobic interaction between the excess of uncomplexed PPO blocks. In  
185 the case of the reverse poloxamines,  $\alpha$ -CD threads their inner PEO blocks. A few CD moieties  
186 (i.e. lower 904/ $\alpha$ -CD ratios, left hand side of the diagrams) suffice to disentangle the octablock  
187 chains to yield structures that gellify by hydrophobic interactions between PPO blocks. This is  
188 indeed the case for reverse Pluronics (PPO-PEO-PPO triblock copolymers) (Larrañeta and Isasi,  
189 2012).

190 An additional consideration regarding the preparation of the samples is needed. Once the  
191 mixtures have been heated to analyze their sol-gel behaviour, they were cooled down to room  
192 temperature. In some instances, a second heating scan yielded different results, producing gel-like  
193 mixtures instead of fluid sols (see Figure A3, Supplementary data). Nevertheless, these  
194 thickening effects can be considered quite subtle for 904 gels and they are not too significant for  
195 90R4 gels either.

196           When aqueous 90R4 or 904 octablock copolymers are mixed with  $\beta$ -CD solutions, no gels  
197 are obtained but solid precipitates. As we have recently shown, the combination of PEO/PPO  
198 linear triblock copolymers (i.e. poloxamers or Pluronics) with  $\beta$ -CD leads also to the formation of  
199 a crystalline precipitate powder (Larrañeta and Isasi, 2012).

200           In contrast to the gel formation observed for  $\alpha$ -CD and PEO/PPO copolymers, the mixing  
201 of the latter with  $\beta$ - or  $\gamma$ -CD aqueous solutions yields white precipitates with a certain crystalline  
202 order (Harada et al., 1995, Li et al., 2001). According to the literature, these precipitates are  
203 formed by the inclusion of the PPO blocks inside the  $\beta$ -CD cavities producing a  
204 polypseudorotaxane type structure (Harada, 1998) yielding self-associated structures such as  
205 nanoplatelets (Perry et al., 2011, Tsai et al., 2010).

206           Although colloidal at first, the  $\beta$ -CD/Tetronic aggregates coalesce in a few minutes. The  
207 kinetics of the process depends on the poloxamine and it is faster for the 90R4 (see  
208 Supplementary data, Figure A4). In the case of 904/ $\beta$ -CD, the PPO blocks are located in the inner  
209 part of the poloxamine arms, so  $\beta$ -CD needs an additional time to pass through the PEO external  
210 blocks. These complexes present a crystalline character that can be evaluated by means of X-ray  
211 diffraction (see Supplementary data, Figure A5). Using NMR and elemental analysis, it has been  
212 found that they possess a defined stoichiometry of about two propylene oxide units per CD  
213 molecule (see Supplementary data, Table A1), an optimal value already reported for other PPO  
214 polymers and copolymers (Harada et al., 1995). The characterization study of these complexes  
215 can be found in the Supplementary data section.

216           When PEO/PPO block copolymers and CDs are combined, different associated systems  
217 can be obtained. Using  $\alpha$ -CD, two types of associations are present in the mixture: (1)  
218 hydrophobic interactions between PPO uncomplexed blocks, and (2) associations between  $\alpha$ -CD  
219 moieties encapsulating PEO complexed blocks. As a result, firm gels are produced (Larrañeta and  
220 Isasi, 2012, Ni et al., 2009). In contrast, the hydrophobic interactions are not present in the

221 mixtures of these PEO/PPO copolymers with an excess of  $\beta$ - or  $\gamma$ -CD because the PPO blocks are  
222 complexed and the hydrophilic PEO blocks do not self-associate in aqueous solutions (Li et al.,  
223 2006).

224 Thus, it becomes evident that the type of cyclodextrin determines the gel character of the  
225 aggregates. Despite of their unfavourable molecular architecture, the complexes of these star-like  
226 copolymers obtained using  $\beta$ -CD are not gelatinous but crystalline precipitates. In contrast, in the  
227 case of  $\alpha$ -CD/Tetronic systems, the resulting hydrogels can be suitable for sorption and delivery  
228 applications. Obviously, these non-covalent interactions can be easily disrupted when the gels or  
229 the complexes are in contact with a large amount of water (Li et al., 2001). This behaviour can be  
230 proved to be very useful for some applications such as drug release because the gel matrix will be  
231 totally eroded in a short period of time. Prior to testing these gels as protein delivery matrices,  
232 their physical properties need to be discussed.

233 **Physical properties of poloxamine/ $\alpha$ -cyclodextrin gels.** The rheological behaviour of  
234 poloxamine/ $\alpha$ -CD mixtures has been analyzed using a rotational viscometer. All the samples  
235 contain 10% (w/w) of  $\alpha$ -CD and different concentrations of reverse 90R4 and direct 904  
236 Tetronic. We have chosen this  $\alpha$ -CD composition as a standard value, between the solubility limit  
237 of the cyclodextrin (14%) and lower compositions which yield mostly solutions instead of gels  
238 (7% and below). These mixtures exhibit a pseudoplastic behaviour, i.e. the viscosity of the  
239 samples decreases when the shear rate is increased (see Supplementary data, Figures A6-A7).  
240 Figure 4 shows the viscosities measured at a constant shear rate of  $40 \text{ s}^{-1}$  as a function of the  
241 copolymer concentration for gels containing the same amount of  $\alpha$ -CD (10 wt%). Interestingly,  
242 both plots show a cusp at about 20% (w/w) of the copolymer. This copolymer/CD ratio  
243 corresponds, in both cases, to four CDs molecules per poloxamine molecule, i.e. a single  $\alpha$ -CD  
244 molecule per PEO complexing block. This was also the case for a linear PEO/PPO copolymer,  
245 Pluronic 10R5, that was previously studied by our group (Larrañeta and Isasi, 2012). Once each

246 PEO block is, on average, threaded to one cyclodextrin moiety, the conformation of the Tetronic  
247 arms change, making the self-assembling process feasible. An excess of uncomplexed  
248 poloxamine molecules (higher Tetronic/CD ratios) contributes to an increase in the viscosity of  
249 the mixture. Thus, it becomes evident why more “ordered” structures, corresponding to mixtures  
250 with less than a 20% of the poloxamine, yield lower viscosity values.

251 Although they present the same singular point (cusp), the viscosity range of 90R4 gels is  
252 significantly higher than that of 904 gels. The different distribution of the blocks in both  
253 copolymers can explain this difference. For 90R4, the outer PPO blocks can establish PPO-PPO  
254 intermolecular interactions easily, contributing to a remarkable increase in the viscosity of the  
255 bulk. On the other hand, the 904 inner PPO blocks can also establish hydrophobic intermolecular  
256 interactions, but in a more restrained way.

257 A comparison between XRD patterns for different 904/ $\alpha$ -CD and 90R4/ $\alpha$ -CD gels with  
258 the polyrotaxane complex formed between a PEO homopolymer ( $M_w= 400$ ) and  $\alpha$ -CD is shown  
259 in Figure 5. The inclusion complex formed between pure PEO and this cyclodextrin yields a  
260 white powder with a channel type structure for  $\alpha$ -CD (Harada et al., 1992). This structure is  
261 clearly detected in the XRD pattern with a characteristic peak located at about  $20^\circ$ , and  
262 corresponds to the hexagonal packing (210) of the channel structure (Chung et al., 2007). Figure  
263 5 shows that the same peak is observed for both 904 and 90R4 gels. Although the amorphous  
264 halo is, in both cases, considerably large, the diffraction peaks show that there is some degree of  
265 order in the gel structure, attributable to the formation of EO/ $\alpha$ -CD complexes that become  
266 aggregated into ordered domains. It has to be pointed out that both gels were prepared using 25%  
267 (w/w) of the copolymer and 10% (w/w) of  $\alpha$ -CD, so there is, on average, less than one CD per  
268 PEO block in both cases. Thus, the hexagonal packing of the CD units detected by XRD implies  
269 side-to-side packing of single rotaxane structures.

270 The mobility of the chain segments within the block copolymers can be modified because  
271 of their complexation with the cyclodextrin moieties (Ni et al., 2009). Table 1 shows the glass  
272 transition temperatures for complexes of 904 and 90R4 with  $\alpha$ -CD for different poloxamine/CD  
273 ratios. As can be seen, the complexes formed between 90R4 and  $\alpha$ -CD do not behave in the same  
274 way as 904/ $\alpha$ -CD ones. It was previously shown that only mixtures formed between linear  
275 PEO/PPO copolymers and  $\alpha$ -CD corresponding EO/CD mole ratios above 2 show a defined  $T_g$   
276 (Ni et al., 2009),(Larrañeta and Isasi, 2012). This characteristic value corresponds to the two EO  
277 units that fit inside a CD cavity. When the EO/CD ratio is below 2, all the PEO blocks are  
278 threaded within CD cavities. These complexed blocks tend to aggregate into ordered domains so  
279 the mobility of the chains is very restricted. Table 1 shows that, for EO/CD ratios higher than 2.7,  
280 Tetronic 904 complexes show a defined  $T_g$ , similar to that of the pure poloxamine. No glass  
281 transitions are detected for mixtures below this ratio (i.e. those with an excess of CD moieties). In  
282 contrast, Tetronic 90R4 samples show a clear  $T_g$  (with a value close to that of the pure  
283 poloxamine) for all the studied EO/CD ratios. Because of their molecular architecture, the  
284 complexation of the PEO blocks of 904 is easier than that of 90R4 PEO blocks, located in the  
285 inner part of the poloxamine arms. For steric reasons, the reverse poloxamine PEO blocks cannot  
286 be fully complexed with CD molecules, so their mobility is not totally restricted and a glass  
287 transition is detected.

288 **Erosion and BSA release kinetics of 90R4/ $\alpha$ -CD gels.** According to the results shown above,  
289 self-assembled hydrogels formed with reverse Tetronic 90R4 and  $\alpha$ -CD can be considered as  
290 potential matrices for drug delivery systems. These gels showed an appropriate consistency, so  
291 erosion kinetic studies were carried out in order to prove if the gels were suitable as release  
292 matrices for sustained delivery purposes. On the other hand, the gels based on the direct  
293 poloxamine (Tetronic 904) result too soft to be used for these purposes, so they were not tested.

294 The release of  $\alpha$ -CD from the gels can be used as an indicator of the erosion kinetics of  
295 the matrix, because  $\alpha$ -CD is one of the components of the gel structure. Figure 6a displays two  
296 erosion kinetic curves at physiological pH and temperature for two different 90R4/ $\alpha$ -CD gels.  
297 One of them (TR15a10), showing a sustained slow erosion kinetics, is composed of 15% of 90R4  
298 and 10% (w/w) of  $\alpha$ -CD. The second one (TR25a10) was prepared using a higher amount of the  
299 poloxamine, 25%, and the same amount of  $\alpha$ -CD, 10%. The latter is more viscous and thicker  
300 than the former, as it was shown in Figure 4, although its erosion is considerably faster. TR15a10  
301 gels contain more CD units per PEO block so the association between complexed PEO blocks is  
302 more important than in TR25a10/CD gels. This gel is thicker because of the hydrophobic  
303 interactions between its PPO blocks, which yield more viscous aggregates. The disaggregation of  
304 polyrotaxane structures in reverse type poloxamers (Pluronics) and poloxamines (Tetronics) is  
305 not as fast as the rupture of PPO-PPO interactions because the cyclodextrin molecules must slide  
306 across the copolymer chain to break the complexes. Consequently, the erosion kinetics of  
307 TR25a10 gels is significantly faster than that of TR15a10.

308 The disaggregation behaviours in different pH media were studied for the same gel  
309 (Figure 6b). The erosion kinetics of three different samples of TR25a10 were evaluated in acidic,  
310 neutral and basic solutions. The dissolution of the gels at pH 1 and 7 is nearly the same, despite  
311 the fact that Tetronic molecules are protonated at acidic pH, so the interactions between Tetronic  
312 molecules should change (Gonzalez-Lopez et al., 2008). Nevertheless, given the size of the  
313 poloxamine molecules, it seems to be quite a small effect in this case. In contrast, the sample  
314 shows a faster erosion process at pH 13. The main reason for this significantly behaviour may be  
315 the ionization of the CD (Maeztu et al., 2011). At basic pH, the external hydroxyl groups of the  
316 CD molecules are deprotonated. For the PEO complexed blocks, the cyclodextrin rings can start  
317 to repel each other, so the stability of the rotaxane clusters decreases.

318           The erosion kinetics is almost the same at acidic and neutral pH: by tuning the hydrogel  
319 compositions, the dismantling process can occur between two and five hours. A sustained drug  
320 release is a promising feature of these systems which should be studied further. To conclude this  
321 investigation, we decided to test the release of bovine serum albumin (BSA) as a model protein in  
322 simulated physiological conditions. Figure 7 shows the release of BSA from TR25a10 and  
323 TR15a10 gels as a function of time, at pH 7 and 37 °C. The release process of BSA from T25a10  
324 is faster than that of T15a10. This is consistent with the gel erosion profiles. As can be seen, both  
325 profiles, i.e. those of the released molecule (BSA) (Figure 7) and the erosion ones (obtained by  
326 measuring the  $\alpha$ -CD concentration in the aqueous release medium) (Figure 6a), are very similar.  
327 This indicates that the release of substances from these gels is dominated by the erosion of the gel  
328 matrix. As it was expected, a large molecule such as BSA (ca. 66.5 kDa) is mainly released as the  
329 assembled CD/poloxamers structures are dismantled.

330

## 331 **CONCLUSIONS**

332 Both poloxamines, direct and reverse, can produce supramolecular gels when interacting with  $\alpha$ -  
333 CD in aqueous media. The comparison between the erosion profiles and the BSA release kinetics  
334 for 90R4/ $\alpha$ -CD gels proves that they can be applied for sustained release during short periods of  
335 time. Although Tetronic 904 gels (i.e. the direct poloxamine) are not firm enough for this study,  
336 they could be used for other types of release matrices applications such as ointments. Taking into  
337 account that both PEO/PPO copolymers and  $\alpha$ -CD seem to be biocompatible, these gels can be  
338 promising for biomedical applications.

339           Besides, it has to be remarked that the viscosity and the gel-to-sol transition temperatures  
340 of these mixtures can be tailored by varying the poloxamine/ $\alpha$ -CD ratios, so they can be tuned to  
341 change at physiological temperatures (see Figures 2 and 3), in order to be applied for *in situ*

342 gelling. An additional important consequence is that the erosion kinetics of the gel, i.e. its  
343 disaggregation speed, can be also tuned by varying the copolymer/ $\alpha$ -CD ratio.

344

#### 345 **ACKNOWLEDGEMENTS**

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349 Complutense) for her help with the NMR results and to C. Cesteros (Universidad del País Vasco  
350 UPV/EHU) for DSC measurements.

351

#### 352 **APPENDIX A: SUPPLEMENTARY DATA**

353 Figures A1–A7 and Table A1.

354

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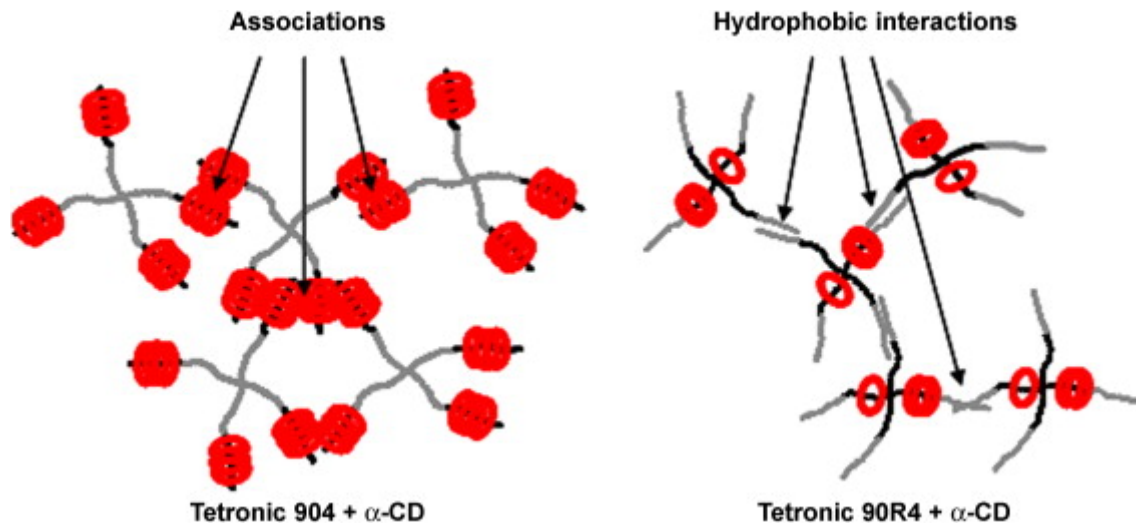


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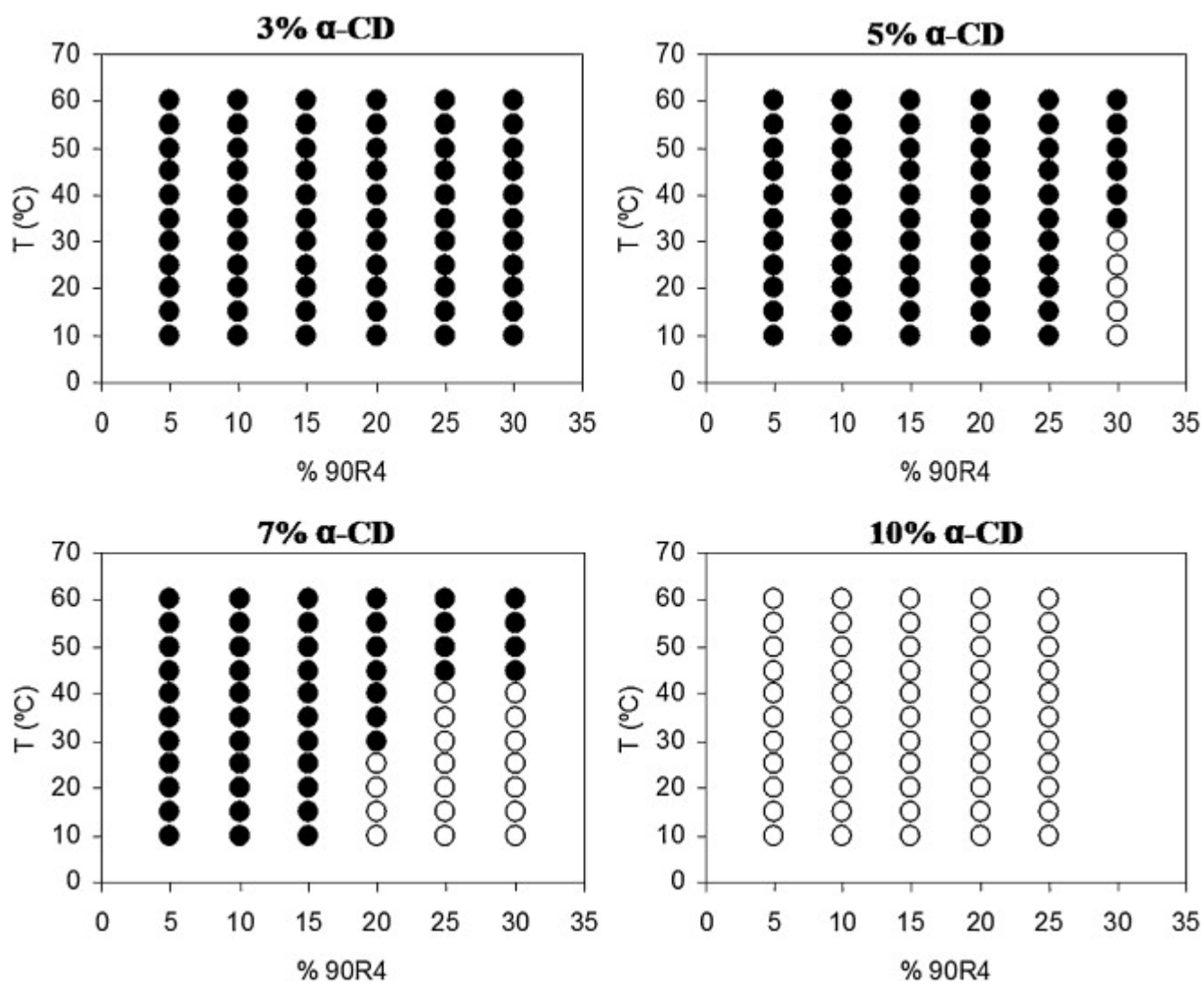
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456 **Figure 1.** Associations between  $\alpha$ -CD (0) and normal 904 or reverse 90R4 Tetronics.

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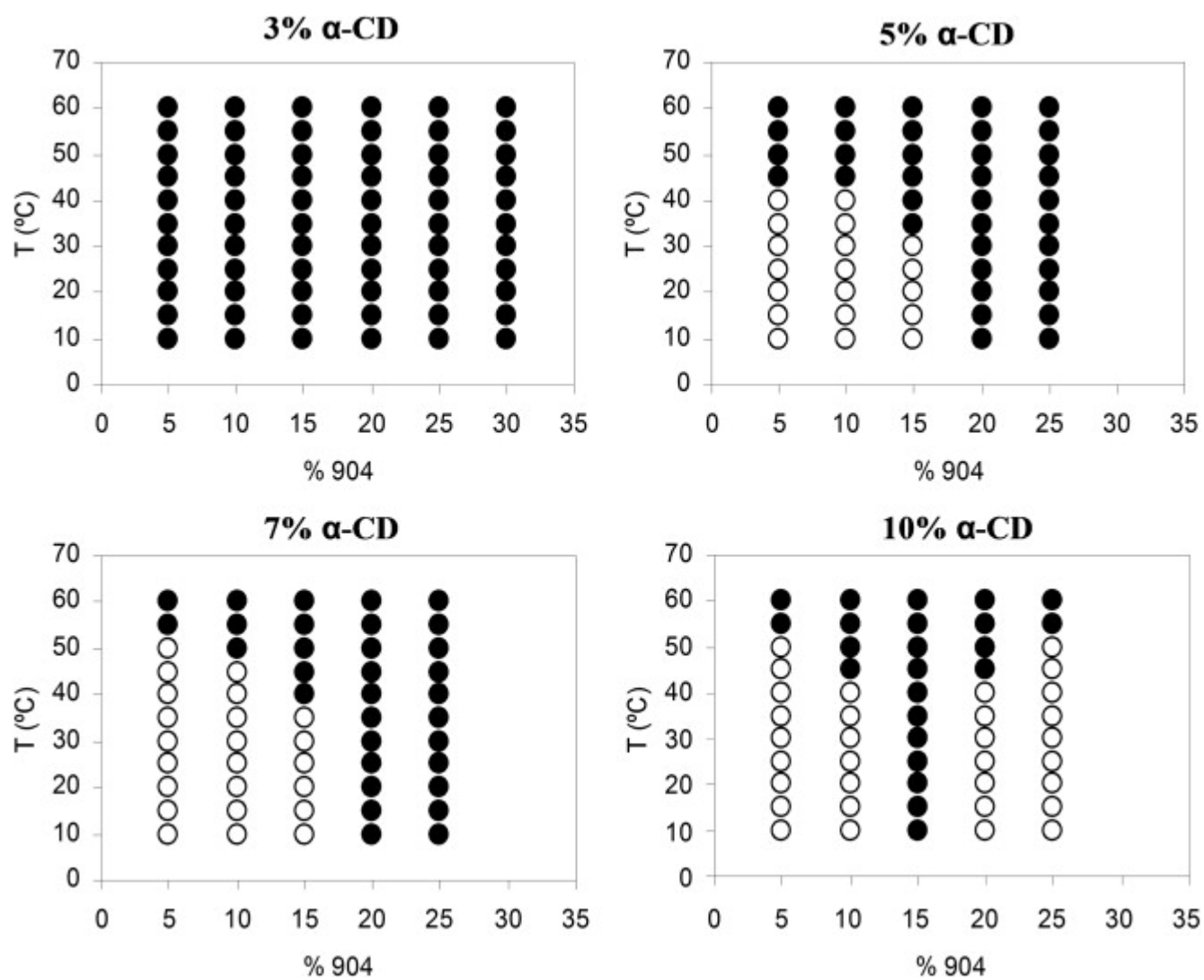


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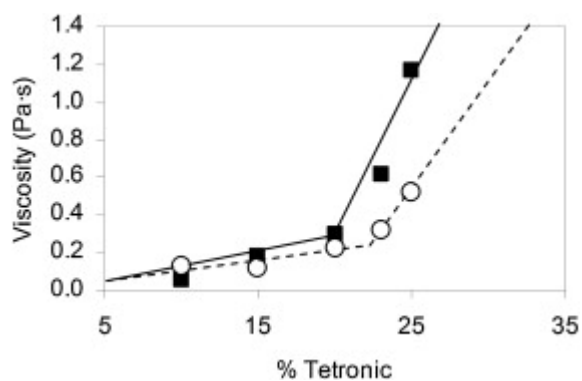
459 **Figure 2.** Temperature–concentration phase diagrams of aqueous reverse Tetronic 90R4 in the

460 presence of  $\alpha$ -CD. Sol phase (●) Gel phase (○)

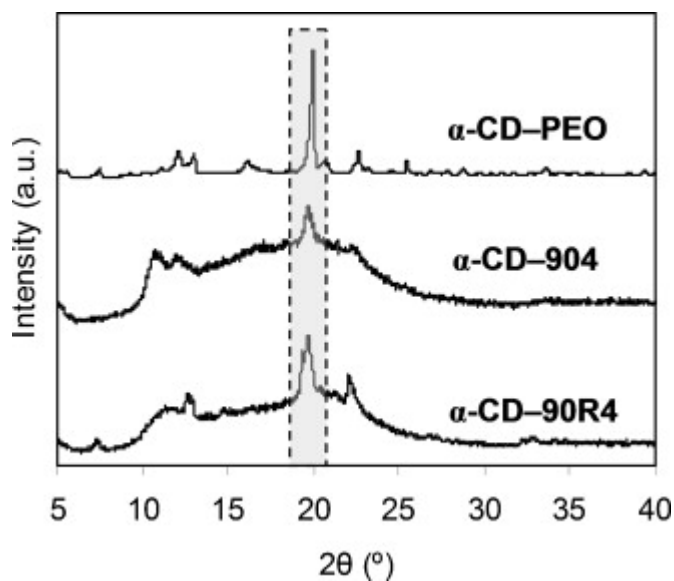
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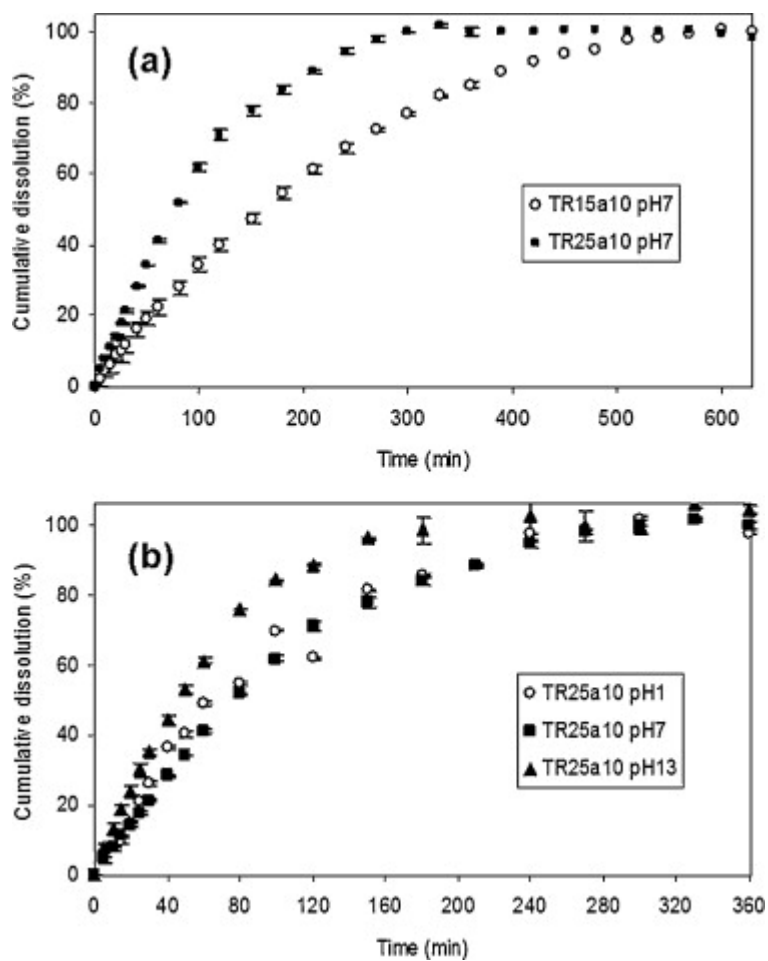
462  
 463 **Figure 3.** Temperature–concentration phase diagrams of aqueous normal Tetronic 904 in the  
 464 presence of  $\alpha$ -CD. Sol phase (●) Gel phase (○).



465  
 466 **Figure 4.** Viscosities of gels with 10% (w/w) of  $\alpha$ -CD and different concentrations of Tetronic  
 467 904 (○) and Tetronic 90R4 (■) measured at a constant shear rate of 40 s<sup>-1</sup>.  
 468



469  
 470 **Figure 5.** Wide-angle X-ray diffractograms for  $\alpha$ -CD-PEO ( $M_w = 400$ ) complex,  $\alpha$ -CD-904 gel  
 471 containing 10% (w/w) of CD and 25% of 904, and  $\alpha$ -CD-90R4 gel containing 10% (w/w) of CD  
 472 and 25% of 90R4.



473

474

**Figure 6.** Erosion kinetics at 37 °C of two mixtures of 90R4 and  $\alpha$ -CD, both containing 10%

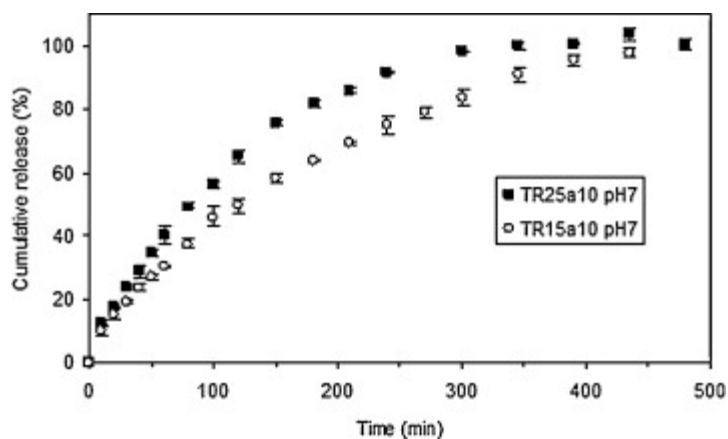
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(w/w) of  $\alpha$ -CD and 25% (w/w) (■) or 15% (w/w) (○) of 90R4 at pH 7 (a), and three TR25a10

476

gels at different pHs: 1 (○), 7 (■) and 13 (▲) (b) (error bars for two replicates).





477  
 478 **Figure 7.** BSA release from two gels, both containing 10% (w/w) of  $\alpha$ -CD and 25% (w/w) (■) or  
 479 15% (w/w) (○) of 90R4 at physiological conditions (error bars for two replicates).

480

481 **Table 1.**  $T_g$  for different complexes of 904 or 90R4 with  $\alpha$ -CD.

482

Sample name	Mixture composition (%)				$T_g$ (°C)
	Copolymer	$\alpha$ -CD	H <sub>2</sub> O	EO/CD	
$\alpha$ -CD	—	—	—	—	—
T1a10	1.00	10.00	89.00	0.94	—
T2a10	2.00	10.00	88.00	1.90	—
T3a10	3.00	10.00	87.00	2.73	—
T4a10	4.00	10.00	86.00	3.58	-56.5
T10a10	10.00	10.00	80.00	9.04	-55.4
T20a10	20.00	10.00	70.00	18.08	-59.0
904	100	0	0	—	-58.3
TR1a10	1.00	10.00	89.00	0.94	-66.1
TR2a10	2.00	10.00	88.00	1.90	-60.1
TR3a10	3.00	10.00	87.00	2.73	-61.3
TR4a10	4.00	10.00	86.00	3.58	-59.8
TR10a10	10.00	10.00	80.00	9.04	-64.7
TR20a10	20.00	10.00	70.00	18.08	-65.6
90R4	100	0	0	—	-62.8

483

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### Table of Contents Graphic

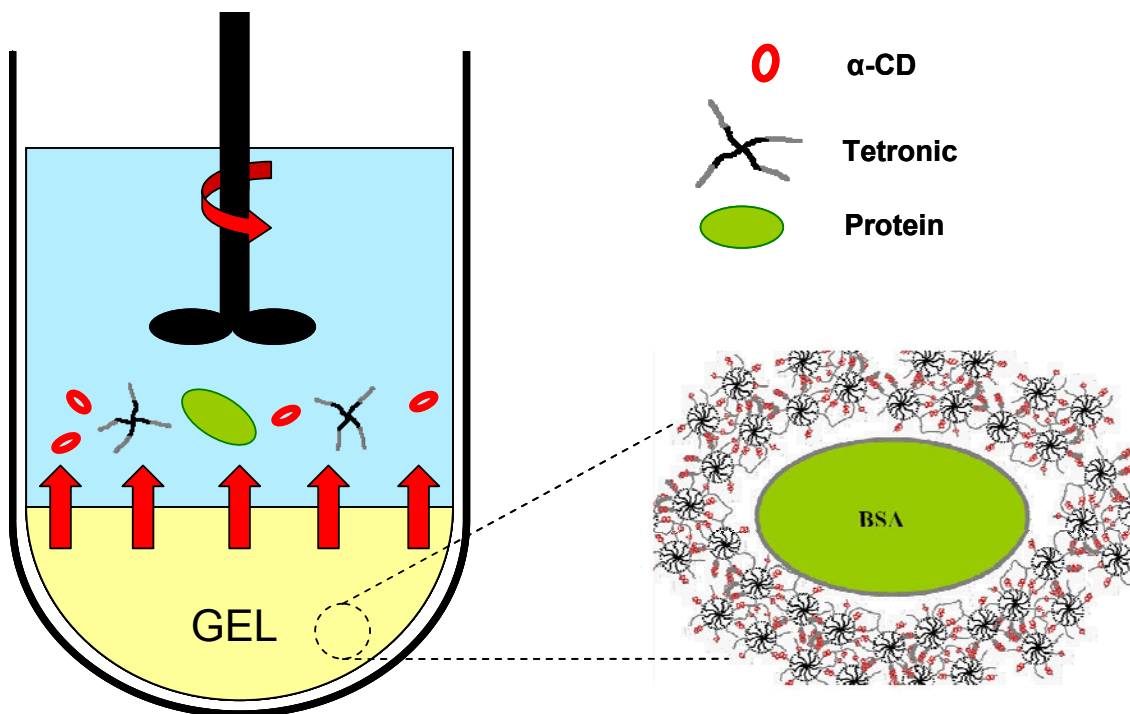
486 *Title: Non-covalent hydrogels of cyclodextrins and poloxamines for the controlled release of*  
487 **proteins**

488 *Authors: E. Larrañeta, J.R. Isasi*

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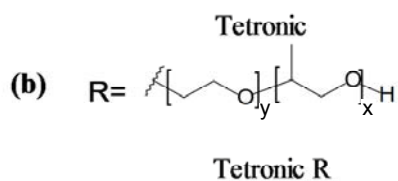
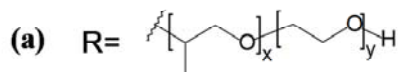
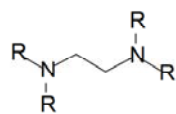
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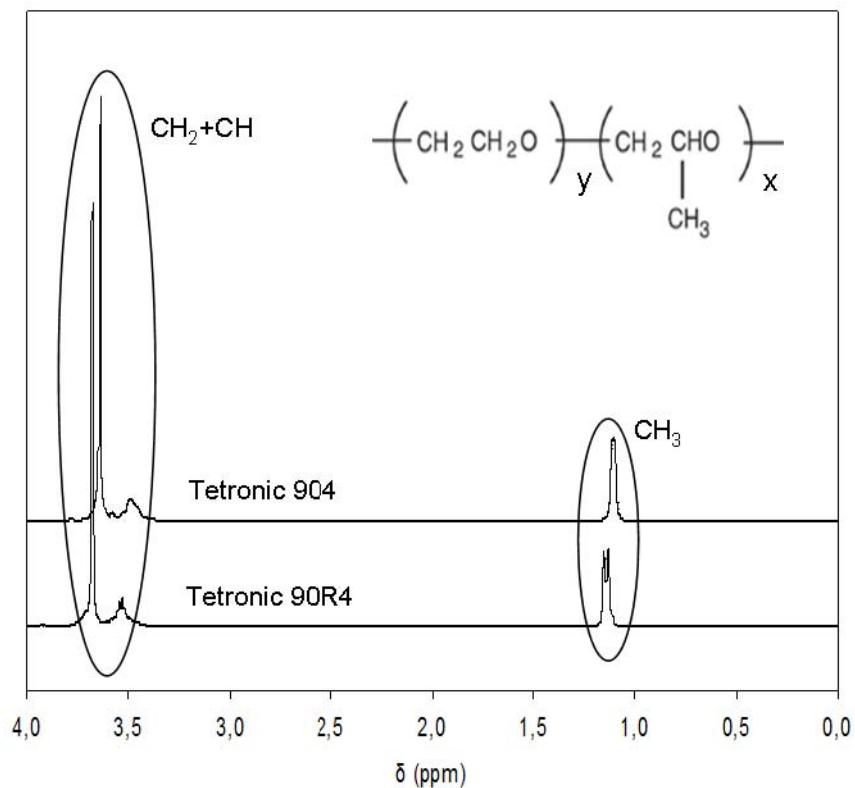
499 **APPENDIX A: SUPPLEMENTARY DATA**

500  
 501 **Title: Non-covalent hydrogels of cyclodextrins and poloxamines for the controlled release of**  
 502 **proteins**

503 **Authors: E. Larrañeta, J.R. Isasi**



504



505  
 506

507 **Figure A1.** <sup>1</sup>H-NMR spectra of Tetronic 904 and Tetronic 90R4. For each copolymer, *x* and *y* values are  
508 calculated using the molecular weight given by the manufacturer, the molecular weights of both units, and  
509 the ratio of both NMR signals.

510

## 511 **Determination of released amounts of BSA**

512 To determine the amount of BSA released three calibration curves are needed:

513 Fluorescence:  $I_f = K_f^{(BSA)} \cdot [BSA]$

514 UV-vis:  $A^{(BSA)} = K_{UV}^{(BSA)} \cdot [BSA]$

515  $A^{(90R4)} = K_{UV}^{(90R4)} \cdot [90R4]$

516 The samples collected by the dissolution testing device were evaluated both by **UV-vis at 280**  
517 **nm** and by **fluorescence emission at 341 nm** (excitation at 280 nm). With these data, the  
518 following equations are obtained:

519 **UV-vis:**  $A_{\text{sample}} = K_{UV}^{(BSA)} \cdot [BSA] + K_{UV}^{(90R4)} \cdot [90R4]$

520 **Fluorescence:**  $I_f/I_Q = K_Q \cdot [90R4] \rightarrow I_Q = I_f / (K_Q \cdot [90R4])$

521 where **K<sub>Q</sub>** is the quenching Stern-Volmer constant at room temperature and **I<sub>Q</sub>** is the actual  
522 intensity measured for each sample and **I<sub>f</sub>** would be that in the absence of the quencher, i.e. the  
523 one we have in the calibration curve.

524 Using the constants from the UV-vis calibration curves ( $K_{UV}^{(BSA)}$  and  $K_{UV}^{(90R4)}$ ) and, in the  
525 case of the fluorescence signal expression, substituting  $I_f$  from its calibration, we obtain two  
526 equations with two unknowns ([BSA] and [90R4]):

527

528 **UV-vis:**  $A_{\text{sample}} = K_{UV}^{(BSA)} \cdot [BSA] + K_{UV}^{(90R4)} \cdot [90R4]$

529 **Fluorescence:**  $I_Q = (K_f^{(BSA)} \cdot [BSA]) / (K_Q \cdot [90R4])$

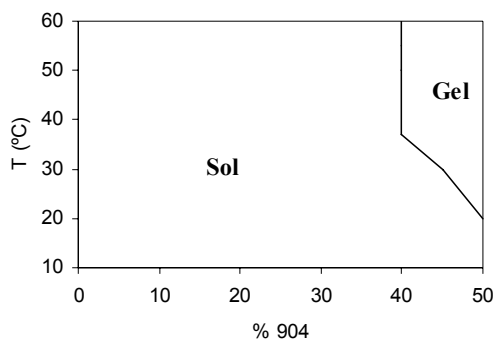
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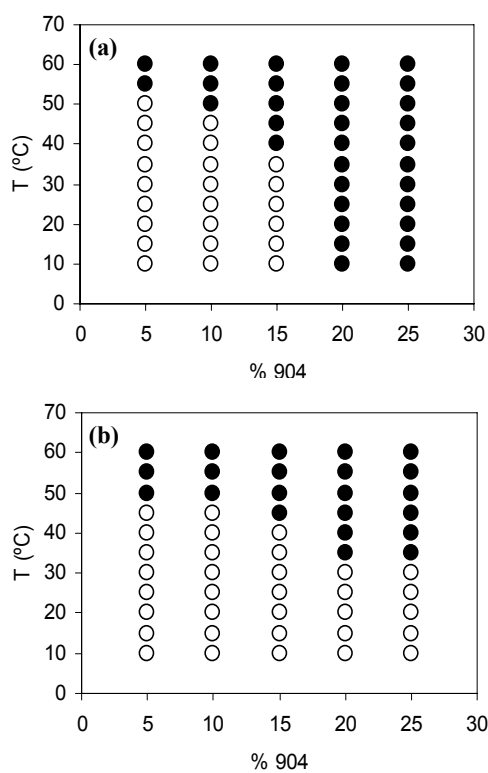
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535 **Figure A2.** Sol-gel phase diagram for 904/water solutions as a function of the temperature.

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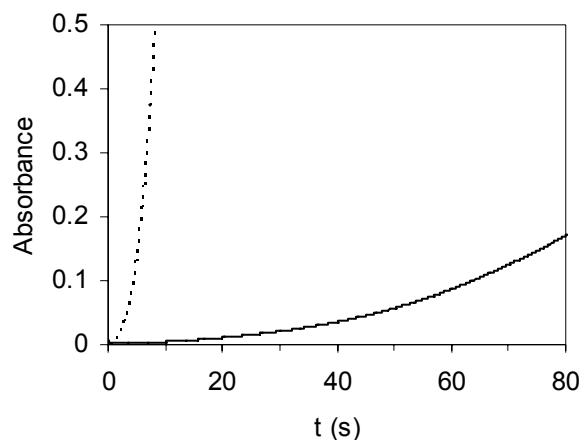


537

538 **Figure A3.** Variation of the temperature-concentration phase diagrams of aqueous Tetronic 904 in the  
 539 presence of 7% of  $\alpha$ -CD. Sol phase (●), gel phase (○). (a) First scan and (b) second scan.

540

541 **Turbidity measurements.** Turbidities of two mixtures containing 0.5% (w/w) of the copolymer (Tetronic  
 542 904 or Tetronic 90R4) and 1.5% (w/w) of  $\beta$ -CD were measured using a JASCO V-630 UV-vis  
 543 spectrophotometer. All the measurements were carried out at a fixed wavelength of 600 nm as a function  
 544 of time.



545  
 546 **Figure A4.** Turbidity of two mixtures containing 0.5% of copolymer, Tetronic 904 (solid line) and  
 547 Tetronic 90R4 (dashed line), and 1.5% of  $\beta$ -CD.

548  
 549 **Characterization of complexes between  $\beta$ -CD and 90R4 or 904**

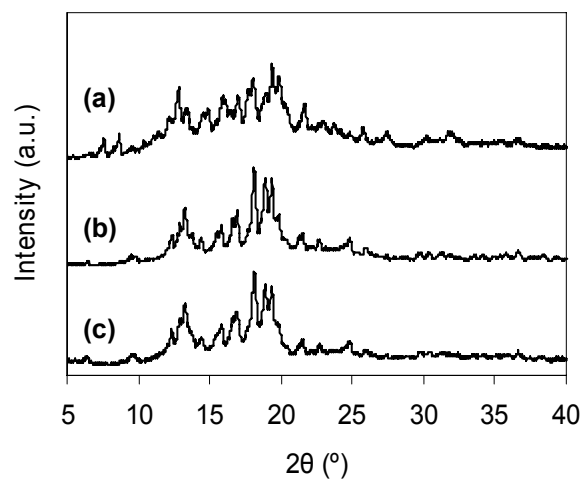
550 **Elemental analysis measurements.** The elemental analysis were carried out with a Leco CHN 900 using  
 551 about 1 mg of sample.

552 **NMR measurements.** The solid-state  $^{13}\text{C}$  CP/MAS NMR spectra were measured using a Bruker AV-400  
 553 NMR Wide Bore with a sample spinning rate of 12 kHz at room temperature. Cross-polarization spectra  
 554 were acquired with a 4  $\mu\text{s}$  proton  $90^\circ$  pulse, a 2.5 ms contact time, and a 5 s repetition time.

555  
 556 **Table A1.** Elemental analysis and NMR calculated PO/CD mole ratios for complexes of 90R4(TR) and  
 557 904(T) with  $\beta$ -CD

sample name	copolymer	mixture composition (%)			elemental composition (%)			NMR PO/CD ratio
		copolymer	$\beta$ -CD	$\text{H}_2\text{O}$	C	H	O	
TR0.5b1.5	90R4	0.50	1.50	98.00	47.65	7.14	45.21	2.47
TR1b1	90R4	1.00	1.00	98.00	47.90	7.14	44.97	
T0.5b1.5	904	0.50	1.50	98.00	45.81	7.04	47.15	2.12
T1b1	904	1.00	1.00	98.00	47.12	7.28	45.60	

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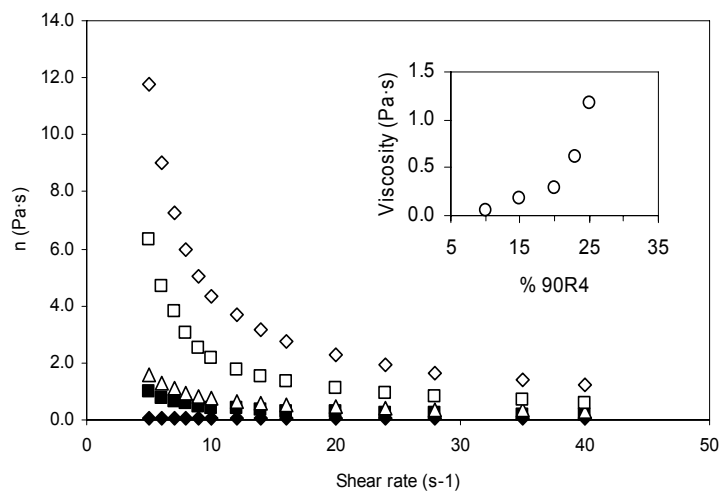


561  
 562 **Figure A5.** X-ray diffraction patterns of:  $\beta$ -CD (a), TR0.5b1.5 (b) and T0.5b1.5 (c).

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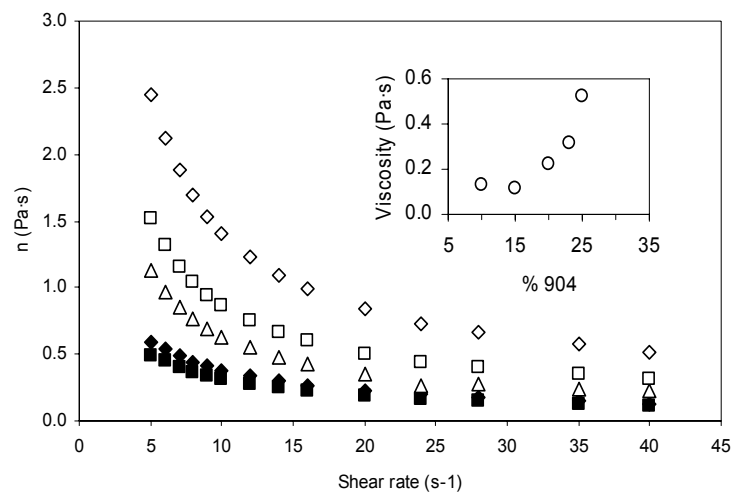
567 **Rheological behavior of  $\alpha$ -CD/90R4 and  $\alpha$ -CD/904 gels**

568  
 569



570  
 571 **Figure A6.** Viscosities of gels with 10% (w/w) of  $\alpha$ -CD and different concentrations of 90R4: 10% ( $\blacklozenge$ ),  
 572 15% ( $\blacksquare$ ), 20% ( $\blacktriangle$ ), 23% ( $\square$ ) and 25% ( $\diamond$ ) as a function of shear rate. The inset shows the dependence of  
 573 the viscosity with the concentration of 90R4 measured at a constant shear rate of  $40\text{ s}^{-1}$ .

574



575  
 576 **Figure A7.** Viscosities of gels with 10% (w/w) of  $\alpha$ -CD and different concentrations of 904: 10% (◆),  
 577 15% (■), 20% (Δ), 23% (□) and 25% (◇) as a function of shear rate. The inset shows the dependence of  
 578 the viscosity with the concentration of 90R4 at a constant shear rate of 40 s<sup>-1</sup>.

579  
 580