



## 29 1. Introduction

30 All areas encapsulating nano- or microparticle-based drug delivery systems have  
31 become some of the most fascinating research areas in modern pharmaceutical development.  
32 Several biodegradable macromolecules such as polyesters, proteins, polysaccharides,  
33 polyelectrolytes, lipids *etc.* (Benkő et al., 2015; Csapó et al., 2016; Danhier et al., 2012;  
34 Kumari et al., 2010; Padilla et al., 2002; Palumbo et al., 2006; Pasqui et al., 2012), or  
35 inorganic materials (layer double hydroxides (LDH), clays, mesoporous silica *etc.* (Deák et  
36 al., 2018; Varga, Benko, Sebok, Bohus, et al., 2014) are used as drug carriers in order to  
37 achieve a targeted drug delivery system and also a controlled drug release process. Generally,  
38 albumin proteins, biocompatible polymers, liposomes, or solid lipid NPs have been utilized  
39 for encapsulation of non-steroidal anti-inflammatory drugs (NSAID) such as ibuprofen,  
40 meloxicam *etc.* (Benkő et al., 2015; Csapó et al., 2016; Varga et al., 2014). Ketoprofen (KP)  
41 also belongs to NSAID and is widely used to treat postoperative pain, including patients after  
42 a gastric resection.

43 Hyaluronic acid (HyA) is a well-known linear polysaccharide of alternating units of  $\beta$ -  
44 1,4-*D*-glucuronic acid and  $\beta$ -1,3-*N*-acetyl-*D*-glucosamine. (Berkó et al., 2013; Bodnár et al.,  
45 2009; Hashad et al., 2017; Lee et al., 2015; Maroda et al., 2011; Wang et al., 2017) Because  
46 of the biocompatible, biodegradable, non-toxic, non-immunogenic and non-inflammatory  
47 features this biomaterial is an ideal candidate for several medical and pharmaceutical  
48 applications. At physiological conditions (pH, ionic strength) the HyA molecules have a  
49 negative charge (hyaluronate form) which results in an extremely high hydrophilic property.  
50 Thanks to the hydrophilic character of HyA it is present in several biological fluids, the  
51 highest amount can be found in the extracellular matrix of the soft connective tissues. The  
52 main disadvantage of this hydrophilic character is that HyA molecules, without chemical  
53 **structural** modification, cannot be simply used as a carrier. **In most cases chemical preparation**  
54 **routes have been selected in order to synthesize the hydrophobized derivatives of HyA (e.g.**  
55 **biocompatible polymer (HyA/poly(lactic acid) (Mayol et al., 2014)) or different alkyl- and aryl-**  
56 **functionalized derivatives (HyA/decylamine(DA)) (Lee et al., 2015; Vafaei et al., 2016).**  
57 **Some cases the HyA has been used as surface functionalizing agent for preparation of e.g.**  
58 **core-shell type NPs (HyA/Human serum albumin-covered chitosan NPs) via electrostatic**  
59 **adsorption of the negatively charged HyA onto the surface of core NPs which has a positive**  
60 **surface charge (Hashad et al., 2017). Besides these derivatives the cross-linked variants of**  
61 **HyA can also be used to encapsulate different drugs (Berkó et al., 2013; Bodnár et al., 2009;**

62 Maroda et al., 2011). Various techniques have been developed for the production of cross-  
63 linked HyA, but one of the commonly used method is the carbodiimide technique. (Bodnár et  
64 al., 2009; Maroda et al., 2011) During this procedure the covalent cross-linking of the  
65 carboxyl functional groups of HyA molecules is carried out with a diamine in an aqueous  
66 medium at room temperature. The main advantage of this technique is that stable colloidal  
67 particles can be formed in water without the use of any surfactant or other organic solvent.  
68 Another possibility is the chemical modification of the HyA molecules by linking aliphatic or  
69 aromatic functional groups to the previously mentioned carboxyl moiety which gives the HyA  
70 macromolecules hydrophobicity.(Choi et al., 2009) Moreover, the less-known neutralization  
71 of HyA via the formation of electrostatic interactions using positively charged amines  
72 containing long aliphatic chains like cetyltrimethylammonium bromide (CTAB) also results  
73 in HyA particles having hydrophobic nature. (Kargerová et al., 2014; Oueslati et al., 2014;  
74 Sauerová et al., 2015) In previous work by other research groups, the KP was encapsulated in  
75 different polymers (e.g. poly (D, L-lactic acid) (PDLLA) or Eudragit) or alginate and gelatin-  
76 based carriers but the HyA has not been used before for the direct encapsulation of KP  
77 molecules. (Arida & Al-Tabakha, 2007; Del Gaudio, Russo, Rosaria Lauro, Colombo, &  
78 Aquino, 2009; Vučen et al., 2013)

79 In this work hydrophobic KP, as the model drug molecule, has been used to develop  
80 and characterize different types of modified HyA-based systems for controlled drug release.  
81 The cross-linking of HyA has been carried out in aqueous media at different ratios of cross-  
82 linking (50; 75 and 100%). Moreover, the hydrophobized derivatives of HyA have also been  
83 prepared by using CTAB at three different HyA monomer/CTAB mass ratios (1:0.2; 1:0.5;  
84 1:0.8). Besides structural characterizations the drug release process has also been investigated  
85 and the experimental results of different colloidal systems were interpreted. One of the main  
86 motivation of this work was to introduce that the extreme hydrophilic HyA after structural  
87 modifications is applicable for encapsulation of highly hydrophobic KP small molecules  
88 which results in the formation of an effective HyA-based nanosized colloidal systems and a  
89 controlled KP release is also feasible.

## 90 2. Materials and Methods

### 91 2.1 Materials

92 Hyaluronic acid sodium salt (HyA, 200-500 kDa) was obtained from Gedeon Richter  
93 Plc. Ketoprofen (KP;  $C_{16}H_{14}O_3$ ;  $\geq 98\%$ ) and CTAB ( $CH_3(CH_2)_{15}N(Br)(CH_3)_3$ ; 95%), sodium  
94 phosphate dibasic dodecahydrate ( $Na_2HPO_4 \times 12H_2O$ ; 98.5%) and sodium phosphate

95 monobasic monohydrate ( $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ ;  $\geq 99\%$ ) were purchased from Sigma-Aldrich.  
96 Sodium chloride ( $\text{NaCl}$ ; a.r.), from Molar Chemicals, was used to prepare isotonic (150 mM)  
97  $\text{NaCl}$  solution. For the cross-linking reaction 2,2'-(ethylenedioxy)bis(ethylamine) (**EDEA**;  
98  $\text{NH}_2\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{NH}_2$ ; 98%) and 1-[3-(dimethylamino)propyl]-3-  
99 ethylcarbodiimid methiodide (**EDC methiodide**;  $\text{C}_2\text{H}_5\text{N}=\text{C}=\text{N}(\text{CH}_2)_3\text{N}(\text{CH}_3)_3\text{I}$ ) were obtained  
100 from Sigma-Aldrich. Highly purified water was obtained by deionization and filtration with a  
101 Millipore purification apparatus (18.2  $\text{M}\Omega \cdot \text{cm}$  at 25 °C). All solvents and reagents used were  
102 of analytical grade and no further purification were made.

## 103 **2.2 Experimental procedures of the HyA modifications**

104 Cross-linked (cl) HyA derivatives were prepared according to the previously published  
105 procedure reported by Bodnár *et al.* (Bodnár *et al.*, 2009) The synthesis was performed at  
106 room temperature. **Namely**, 200 mg HyA was dissolved in water to produce 1 mg/mL solution  
107 then the pH was adjusted to pH = 5.5. The stoichiometric ratio of cross-linking was 50%, 75%  
108 and 100% resulting in cl-HyA/50%, cl-HyA/75% and cl-HyA/100% samples. Accordingly,  
109 1.88 mL, 2.82 mL and 3.76 mL **EDEA** solution (1 v/v%, pH = 5.5) was added to the HyA  
110 solution and mixed for 30 min. Then 80 mg, 120 mg and 160 mg **EDC methiodide** was  
111 dissolved in water and added to the mixture drop by drop, respectively. After an overnight  
112 stirring the product was purified by dialysis for 7 days against distilled water and the aqueous  
113 solution of the final product was freeze-dried. For CTAB modification different calculated  
114 amount of surfactant was added to the aqueous solution of HyA to change the hydrophobicity.  
115 The mixture was stirred for 30 min before further use.

## 116 **2.3 Preparation of KP-containing systems**

117 Because of the low solubility of KP in pure Milli-Q water ( $c_{\text{max}} = 0.051 \text{ mg/mL}$ ) all drug  
118 containing samples were prepared in phosphate buffer solution (PBS) at pH = 7.4 at 25 °C  
119 using constant ionic strength (0.9 %  $\text{NaCl}$ ) **which highly increased in the KP solubility** ( $c_{\text{KP}} =$   
120  $20 \text{ mg/mL}$ ). In all cases constant KP ( $c_{\text{KP}} = 20 \text{ mg/mL}$ ) and constant HyA concentrations  
121 (100.0-100.0 mg lyophilized **HyA and cl-HyA/mL**) were used. **The aqueous KP solutions**  
122 **were added to the different individual cl-HyA and HyA/CTAB samples which resulted in the**  
123 **formation of a gel-like structure after 24 h. After KP loading the samples were diluted to 0.1%**  
124 **and centrifuged (8000 rpm, 10 min). The supernatant contained only 4.5-5.0 % remained KP**  
125 **molecules determined by the previously registered spectrophotometric calibration curve.**  
126 **According to this determination method the loading efficiency is ca. 95.5-95.0%.**

127

## 128 **2.4 Methods for structural characterizations**

129 High-resolution transmission electron microscopy (HRTEM) images were recorded on a  
130 Tecnai G2 instrument at 200kV accelerating voltage and they were analyzed using ImageJ  
131 software. The particle size and zeta potential values were determined by dynamic light  
132 scattering (DLS) with a Zetasizer Nano ZS ZEN 4003 apparatus (Malvern Ins., UK) equipped  
133 with a He-Ne laser ( $\lambda = 633$  nm). The measurements were performed at  $25 \pm 0.1$  °C, with an  
134 angle detection of  $173^\circ$  in a clear disposable zeta cell. In order to determine the maximum  
135 amount of CTAB to be added prior to precipitation 0.02 M of CTAB was added stepwise (20-  
136 20  $\mu\text{L}$ / step) to 0.2 mg/mL concentration of HyA in PBS and the zeta potential values were  
137 registered with DLS. Turbidity measurements were performed by a Precision Bench Turbidity  
138 Meter LP2000 (Hanna Ins.), while the conductivity was measured by a Radelkis OK- 114  
139 conductometer equipped with an electrode with sheet plates. **The Fourier transform infrared  
140 (FT-IR) spectroscopy studies were performed by using Jasco FT/IR-4700 spectrometer with  
141 ATR PRO ONE Single-reflection accessory (ABL&JASCO, Hungary). Spectra were  
142 registered at  $4\text{ cm}^{-1}$  optical resolution by averaging 256 interferograms.**

## 143 **2.5 Isothermal Titration Calorimetry (ITC) studies**

144 Thermometric titration experiments were performed at 298.15 K with a computer-controlled  
145 VP-ITC power-compensation microcalorimeter (MicroCal) in order to determine the degree  
146 of the charge compensation of the CTAB in presence of HyA. Deionized water or HyA  
147 solution (1.4 mL) in the sample cell was titrated under constant stirring with 300  $\mu\text{L}$  of CTAB  
148 solution in aliquots of 10  $\mu\text{L}$  in periodic time intervals of 5 min. The enthalpograms  
149 (calorimeter power signal vs time) were evaluated by means of Origin Microcal 7.1. software.  
150 **ITC curves were** successfully fitted by using the sigmoidal Boltzmann equation. **The** modified  
151 version of Boltzmann equation has been used to improve the precision of the determination of  
152 the critical micellization concentration (*cmc*).[\(Juhász et al., 2017; Kiraly et al., 2001\)](#)

## 153 **2.6 Rheological studies**

154 All rheological measurements were performed using an Anton Paar Physica MCR 301  
155 Rheometer (Anton Paar, GmbH, Germany) at  $25.0 \pm 0.1$  °C to provide concentration-  
156 dependent structural information on the modified HyA-based drug carrier systems. The  
157 measuring system equipped with a 25 mm diameter parallel cone-plate geometry (CP25-1-  
158 SN12204), a double-gap- (DG26.7-SN12740) and a concentric cylinder geometry (CC27-  
159 SN12793). The rheometer utilized a temperature controlled water bath in combination with a

160 Peltier heating system for accurate control. Detailed parameters of the rheological  
161 measurements, as well as the evaluation process, are summarized in the Supplementary.

## 162 **2.7 *In vitro* drug release experiments**

163 The release rate of KP molecules was determined by spectrophotometric measurements  
164 detected the characteristic absorbance band of KP at 260 nm using a UV-1800 (Shimadzu)  
165 double beam spectrophotometer with a 1 cm quartz cuvette in the range of 200-500 nm. The  
166 *in vitro* drug release experiments were carried out in a phosphate buffer (PBS, pH = 7.4) at 25  
167 °C. A cellulose membrane (Sigma-Aldrich) was used as a dialysis membrane. The release  
168 process was followed for 480 min (8 h). Samples were taken every 10 minutes in the first  
169 hour and then once per hour. Analysis of *in vitro* drug release data helps evaluate the release  
170 kinetics and mechanism. Numerous mathematical models (zero-order, first-order, Weibull,  
171 Hixone-Crowell, Korsmeyere-Peppas, *etc.*) have been used to describe the release properties  
172 of the drug molecules (Costa et al., 2001; Peppas et al., 1977). **The detailed description of the**  
173 **different kinetic models is summarized in Supplementary.** To determine the value of the  
174 kinetic constants and other parameters of the applied release kinetic models, the sum of the  
175 square of differences between the measured and predicted concentration values have been  
176 minimalized using a spreadsheet based computer application for nonlinear parameter  
177 estimation (Juhász et al., 2016; Juhász, Csapó, Vécsei, & Dékány, 2017).

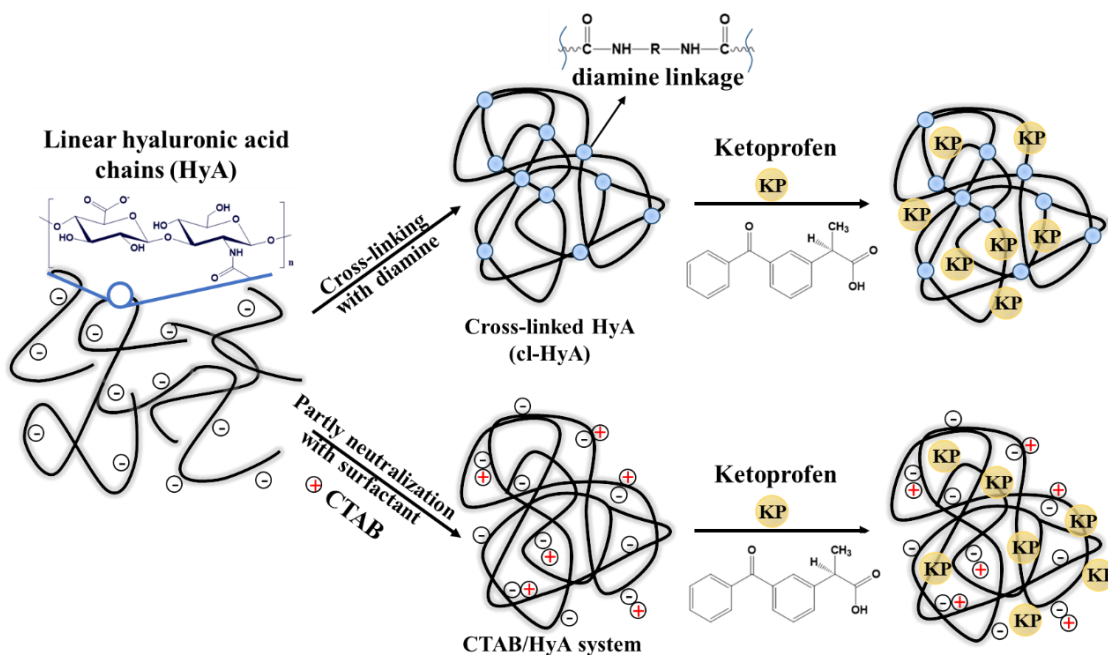
## 178 **3. Results and Discussion**

### 179 **3.1 Structural characterization of the different HyA-based carriers**

180 Due to its highly hydrophilic nature, HyA as a possible carrier for encapsulation of  
181 hydrophobic agents requires structural modifications. Both the cross-linked (cross-linking  
182 ratio of 50, 75 and 100%) and hydrophobized derivatives (partly neutralization with  
183 positively-charged amines) of the HyA carrier have been successfully prepared. **FT-IR**  
184 **studies, which are in good agreement with the previously published data, (Barbucci et al.,**  
185 **2002; Jiang et al., 2015) have been performed to identify the success of the formation of**  
186 **cross-linking, the results are presented in Fig.S1.**

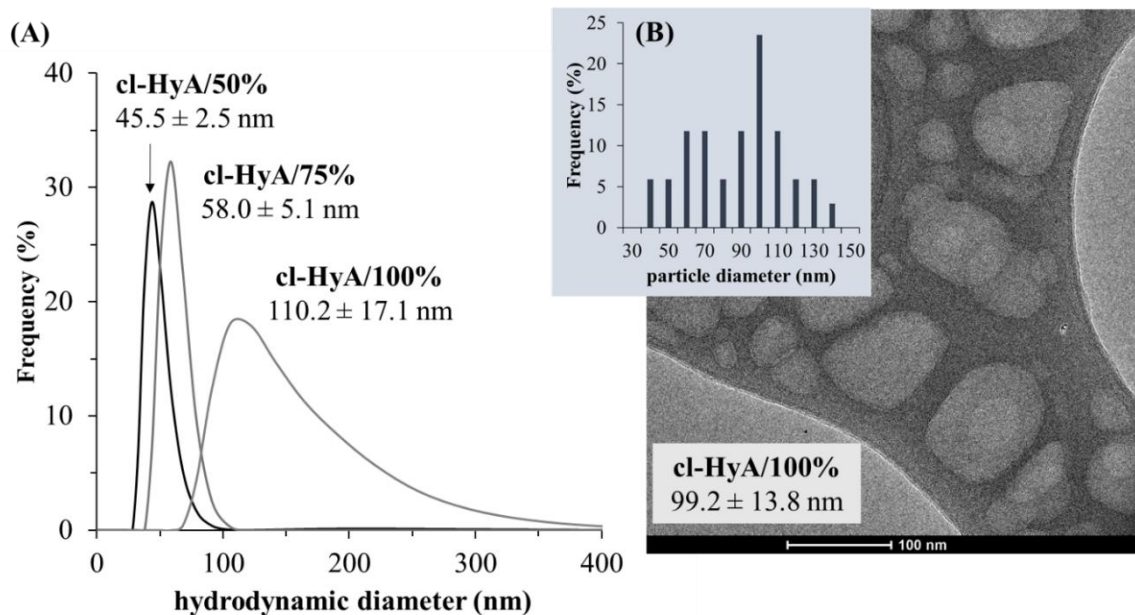
187 **The different HyA modification methods are summarized in Scheme 1.**





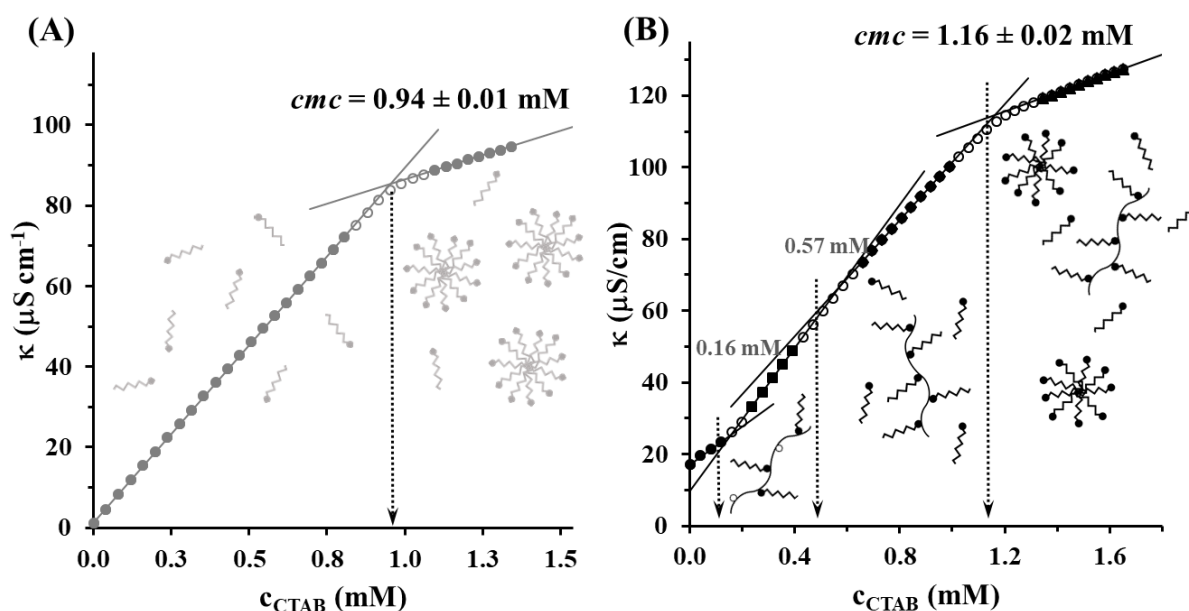
188  
 189 **Scheme 1.** Schematic representation of HyA modifications: cross-linking and neutralization  
 190 with positively-charged amine.

191 **Due to structural modifications it was** found that increasing cross-linking ratio from 50 % to  
 192 100% results in the formation of nanosized particles, with an increasing **size** of *ca.* 45 nm (cl-  
 193 HyA/50%) and *ca.* 110 nm (cl-HyA/100%). **Figure 1.** shows the particle size distributions of  
 194 **cl-HyA** carrier systems determined by DLS with a representative TEM image of the cl-  
 195 HyA/100% particles. The registered TEM image is in accord with the DLS results, the slight  
 196 difference between the results can be explained by the hydrodynamic diameter. However the  
 197 particle size increases with the cross-linking ratio, the presence of more polydispersed  
 198 systems can be confirmed by DLS. In contrast, the measured Zeta-potential values **at neutral**  
 199 **pH (pH ~ 7.0)** shows an increased stability (cl-HyA/50%:  $\zeta = -13.8 \pm 0.1$  mV, cl-HyA/75%:  $\zeta$   
 200 =  $-20.0 \pm 2.1$  mV, cl-HyA/100%:  $\zeta = -23.6 \pm 0.6$  mV) for the application of higher  
 201 concentration of cross-linker. For HyA/CTAB systems the structure, the charge, as well as the  
 202 optimal ratio of HyA and CTAB, were determined by conductometric, turbidity, Zeta-  
 203 potential and ITC measurements in an aqueous solution at 25 °C. It is well known that the  
 204 CTAB molecules are capable of forming micelles when the concentration reaches the *cmc*.



205  
 206 **Figure 1.** Particle size distribution of the different **cl-HyA** carriers determined by DLS (A)  
 207 and a representative HRTEM image and particle size distribution of the **cl-HyA/100%** system  
 208 (B).

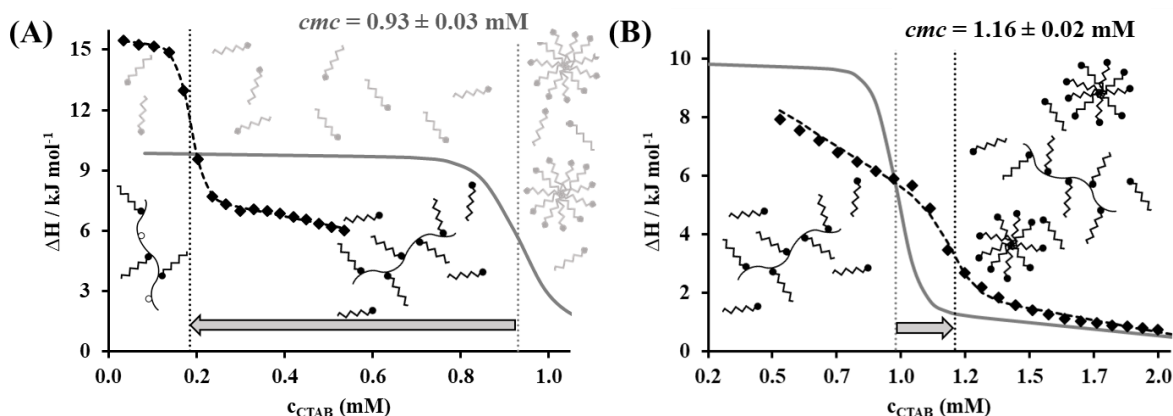
209 According to the parallel conductometric measurements  $0.94 \pm 0.01$  mM of *cmc* value is  
 210 obtained for CTAB (**Figure 2A**), while same  $0.94 \pm 0.01$  mM value was determined by ITC  
 211 as the grey continuous line represents on **Fig. 3.B**.



212  
 213 **Figure 2.** Determination of the *cmc* of CTAB by conductometric measurements in the  
 214 absence (A) and in the presence (B) of HyA ( $c_{\text{HyA}} = 0.1$  mg/mL).

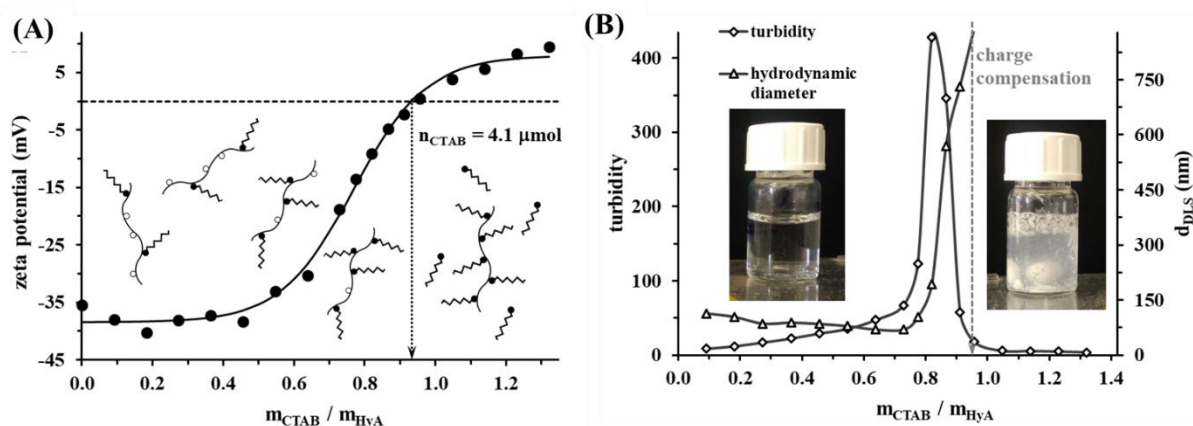


215 Both the conductometric and ITC studies have been carried out in the presence of 0.1 mg/mL  
 216 of HyA and as **Figure 2B** and **Figure 3B** represent, the *cmc* value shifted to  $1.16 \pm 0.02$  mM,  
 217 respectively.



218  
 219 **Figure 3.** Calorimetric enthalpies of dilution obtained from ITC experiments for CTAB in the  
 220 presence of HyA ( $c_{\text{HyA}} = 0.1$  mg/mL) at  $c_{\text{CTAB}} = 12$  mM (A) and  $c_{\text{CTAB}} = 5$  mM (B) at 298 K.

221 The continuous grey lines in **Fig. 3** represent the enthalpogram of pure CTAB in the absence  
 222 of HyA under the same conditions. The degree of this shift (presence of excess CTAB)  
 223 strongly depends on the total amount of HyA in the sample. Calculating with the HyA  
 224 concentration we can conclude that the negatively charged HyA, before the formation of  
 225 micelles, is totally neutralized by CTAB. At the neutralization point the CTAB/HyA  
 226 monomer ratio is *ca.* 1:1 molar (0.96:1.0 mass ratio) obtained by conductometry and ITC  
 227 studies. In order to determine the equivalent charge of the linear HyA, an additional ITC  
 228 measurement was performed with diluted surfactant solution ( $c_{\text{CTAB}} = 5$  mM,  $c_{\text{HyA}} = 0.1$   
 229 mg/mL). As can be seen in **Figure 3A** an inflection point is observed at  $0.2 \pm 0.01$  mM which  
 230 corresponds to nearly 1.2:1.0 surfactant/HyA monomer molar ratio. This observation also  
 231 supports the equivalent charge compensation of HyA monomer with CTAB. **As Fig. 4 shows**  
 232 the Zeta potential of the negatively-charged polymer reaches the zero value at 0.95:1.0  
 233 CTAB/HyA monomer mass ratio which is in accord with the conductometry, and ITC  
 234 experiments. Both the turbidity measurements and the change of the average particle  
 235 diameter of the CTAB/HyA system confirm the aggregation of the polymer chains after the  
 236 charge compensation of the carboxyl groups of HyA.



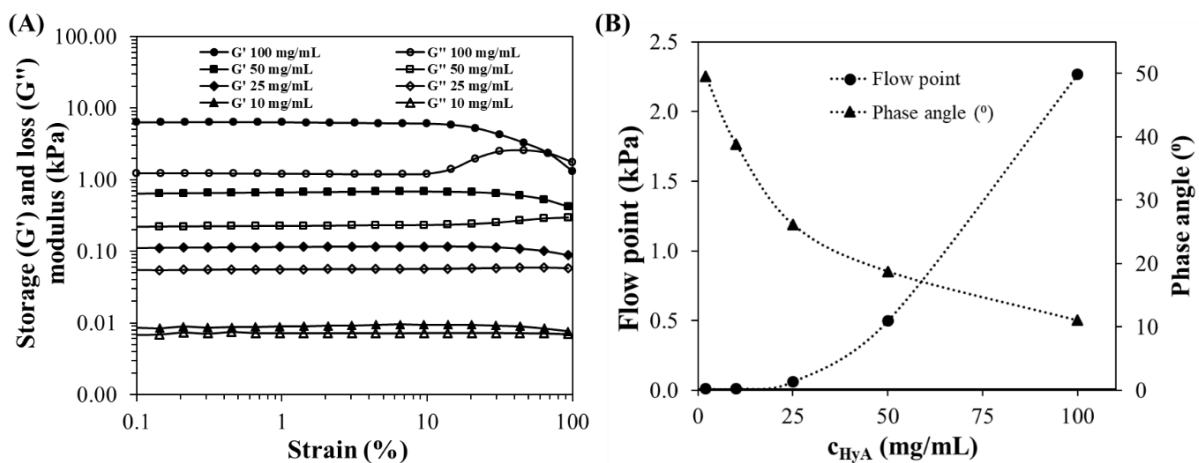
237  
 238 **Figure 4.** Zeta potential (A), turbidity and hydrodynamic diameter (B) of the HyA/CTAB  
 239 system as a function of increasing CTAB/HyA monomer mass ratio ( $c_{HyA} = 0.2 \text{ mg/mL}$ ) The  
 240 (B) represents the photos of the samples before (at  $m_{CTAB}/m_{HyA}=0.2$ ) and after (at  
 241  $m_{CTAB}/m_{HyA}=0.95$ ) charge compensation of HyA.

242 Above this surfactant concentration (0.95 mg CTAB/1.0 mg HyA monomer) the **particle size**  
 243 rises dramatically (from 50 nm to *ca.* 750 nm) while the turbidity decreases due to the  
 244 aggregation of the hydrophobized and neutralized polymer chains. **The registered DLS curves**  
 245 **of HyA/CTAB system at different  $m_{HyA}/m_{CTAB}$  ratios are presented in Fig. S2.**

### 246 3.2 Concentration-dependent rheological characterization of the aqueous HyA solutions 247 and hydrogels before structural modifications

248 Based on the rheological **studies** we found that, the increase in **polymer** concentration resulted  
 249 in a dramatic change in the rheological behavior. **The HyA** solutions show Newtonian, non-  
 250 Newtonian and viscoelastic behavior **as the concentration** increases from 0.05 to 100 mg/mL.  
 251 The viscosities of diluted and moderately concentrated polymer solutions were determined by  
 252 a rotational technique based on the fitting of registered flow curves. In the case of **much** dilute  
 253 solutions, the linear flow curves prove the validity of the Newton law (**Figure S3**). **For these**  
 254 extremely diluted polymer solutions, which are usually considered as a molecular solution,  
 255 **the** viscosity variations are associated with a behavior (deformation, orientation etc.) of  
 256 separate molecules under flow conditions. **Generally,** the viscosities of moderately  
 257 concentrated solutions of high-molecular weight polymers are known to be variable quantities  
 258 which decrease with increasing shear rate. This shear-thinning effect has been understood for  
 259 a long time and **as Fig. S4 shows similar** behavior was observed **for HyA above** 1.00 mg/mL  
 260 concentration. In the case of these concentrated solutions, a varying viscosity is assumed to be  
 261 due to the entanglement of linear polymer chains. An alternative explanation of this effect, is

262 based on an assumption of weak macromolecular cross-linking. These secondary **bindings**  
 263 disappear and reappear again as a result of thermal motion, and the average number of these  
 264 weak cross-links in the shear flow decreases when the shear rate is increased. While this  
 265 characteristic is very desirable, it creates problems when attempting to measure the viscosity  
 266 of HyA solutions and **concentrated** gels. A single-point viscosity test such as that typically  
 267 conducted on a simple viscometer is insufficient to fully characterize the material. Instead of  
 268 this, a viscosity/shear rate profile (such as that shown in **Figure S4**) is more suitable as a  
 269 means of measuring this material. **Moreover**, we report the **results of the dynamic**  
 270 **measurements, which show the change of the loss and the storage modulus ( $G'$  and  $G''$ )** as a  
 271 function of amplitude, under  $10\text{ s}^{-1}$  oscillation frequency. All the samples were tested but  
 272 only some results are presented for clarity. For the linear polymer (**Figure 5**), we observe the  
 273 expected behavior. Polymer gels essentially show elastic character at low strain, while at high  
 274 strain the loss modulus dominates ( $G'' > G'$ ). The distance between the  $G'$ , and  $G''$  curves has  
 275 no such relevance as the ratio  $G''/G'$  which is equal to  $\tan\delta$ , **this parameter** is a measure of the  
 276 internal friction of the material in that condition.



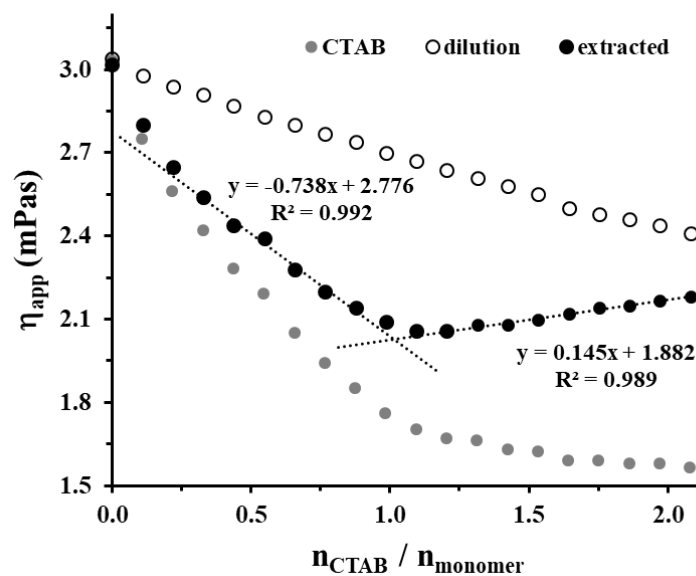
277  
 278 **Figure 5.** Strain (A) and concentration (B) dependence of the viscoelastic parameters (storage  
 279 modulus (solid symbols) and loss modulus (hollow symbols), flow point and phase angle) of  
 280 linear HyA hydrogels.

281 When  $G''$  is higher than  $G'$ ,  $\tan\delta$  is the larger one we can say that the sample is more viscous  
 282 than elastic, and when  $G'$  is higher and  $\tan\delta$  is the smaller one the sample shows an elastic  
 283 property. Amplitude scans of the solvated linear HyA shows that the  $G''/G'$  ratio does not  
 284 change as strain increases from 0.1 to 10.0%, it means that the internal friction is independent  
 285 of strain in this region. Above strain value of **10%**, the samples show non-linear dependence,

286 according to the pseudo-plastic or plastic behavior. The point where  $G''$  crosses  $G'$  is denoted  
 287 as the flow point, and above this strain value the viscous behavior dominates. **Figure 5.B**  
 288 clearly shows that the flow points increase dramatically, while the phase angle decreases as  
 289 the polymer concentration increases. The decrease of the phase angle indicates the change  
 290 from Newtonian behavior to elastic behavior, which confirms viscoelastic fingerprint  
 291 characterizing concentrated HyA solutions with weak gel-like behavior (Iannitti et al., 2013;  
 292 Liang et al., 2007).

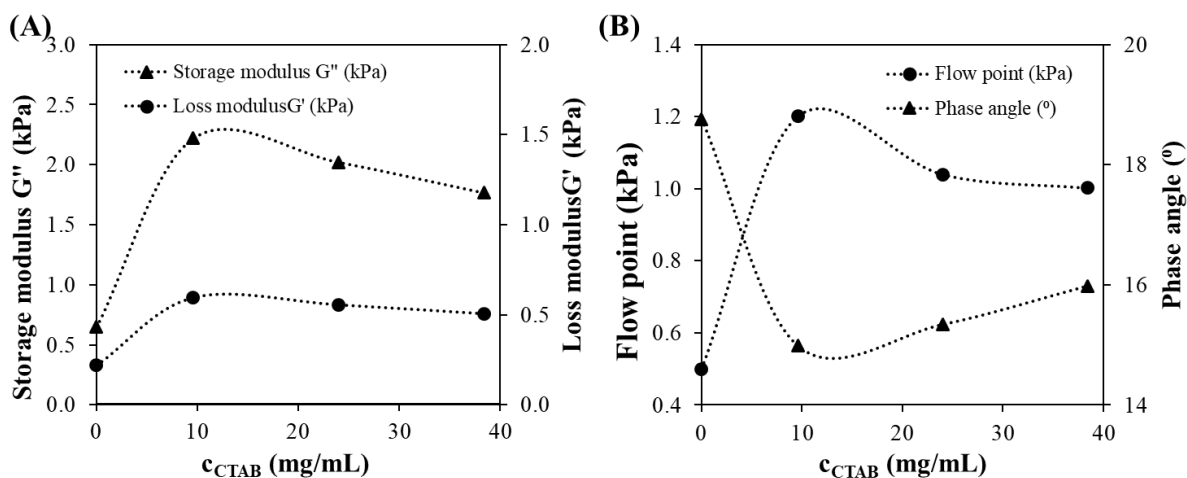
### 293 3.3. Rheological characterization of the hydrophobized CTAB/HyA systems

294 To characterize the hydrophobized form of the polymer, a constant (0.1 mg/mL) initial HyA  
 295 concentration was chosen for the steady shear rate measurements of the CTAB solution  
 296 diluted HyA samples and a larger concentration (50 mg/mL) was applied in the case of  
 297 amplitude sweep investigations. As can be seen in **Figure 6**, the apparent viscosity of the  
 298 polymer solution continuously decreases, due to the added surfactant, and a breakpoint can be  
 299 observed on the viscosity vs. molar ratio ( $n_{\text{CTAB}} / n_{\text{monomer}}$ ) curve. After extraction the dilution  
 300 effect only in the pre-break region can be observed. Above *ca.* 1:1 surfactant/monomer molar  
 301 ratio, the viscosity of the neutralized and thus the hydrophobized polymer solution, shows a  
 302 rising trend. After this observation it can be stated that in addition to conductivity and ITC  
 303 measurement a modified rheological investigation also suitable for detection of the structural  
 304 change of the polymer – surfactant colloid system.



305  
 306 **Figure 6.** Steady shear rate determined apparent viscosity of linear HyA ( $c_{\text{HyA}} = 0.1$  mg/mL)  
 307 titrated with CTAB ( $c_{\text{CTAB}} = 25.0$  mM) solution.

308 As illustrated in **Figure 7**, the varying degrees of hydrophobized polymer based hydrogels,  
 309 show elasticity at low strain, while at high strain range the loss modulus dominates ( $G'' > G'$ )  
 310 as the above reported linear **HyA** hydrogels. As a result of added surfactant at 10 mg/mL  
 311 CTAB concentration (20% of the neutralization needed surfactant amount) the flow point  
 312 increased dramatically and it is slightly reduced by the effect of the following (50% and 80%)  
 313 additional surfactant. The opposite tendency can be observed with regard to the change of  
 314 phase angle value in the function of CTAB concentration. When the concentration of polymer  
 315 is over the 50 mg/mL and surfactant is 10.0 mg/mL or lower the hydrogel becomes more  
 316 elastic than without surfactant while above this surfactant amount the elastic behavior turns  
 317 Newtonian. The same trend was observed in the case of varying degrees of cross-linked  
 318 polymer hydrogels (**Figure S5**.) where the flow point almost reaches the zero value due to the  
 319 structure modification of polymer chains, and the changes of phase angle showed a more and  
 320 more viscous character. The latter outlined two observations confirm the assumption that both  
 321 the added CTAB, and the cross-linking agent break the spontaneously formed coherent  
 322 structure from the solvated biopolymer.



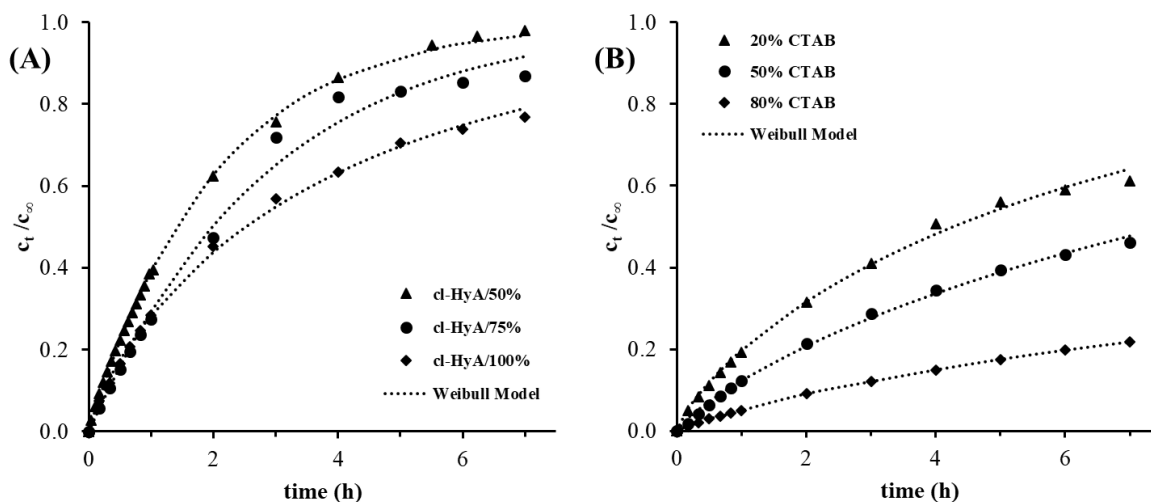
323  
 324 **Figure 7.** Amplitude sweep determined rheological parameters (A: storage modulus and loss  
 325 modulus; B: flow point and phase angle) of linear HyA hydrogels ( $c_{HyA} = 50$  mg/ mL) as a  
 326 function of  $c_{CTAB}$ .

### 327 **3.4 In vitro drug release experiments**

328 The detailed rheological characterization of the pure and modified drug carrier systems  
 329 greatly contributes to the better understanding the mechanism of the drug release process. The  
 330 mechanism of drug release from the HyA-based carriers was investigated using model-  
 331 dependent methods. The drug release results were fitted into first-order kinetics, Korsmeyer–

332 Peppas and Weibull models. Drug release curves of KP from varying degrees of  
 333 hydrophobized or cross-linked polymer-based carriers are depicted in **Figure 8**. As it can be  
 334 seen, all the formulations are able to impede KP release for more than 6 h, but the release of  
 335 the drug from the cross-linked polymer is almost complete within 7 h. In particular, the  
 336 release kinetic of KP from cl-HyA/50% is the fastest and, above all, poorly controlled. By  
 337 adding the linker from 50% to 75 and 100% into the polymer it was possible to achieve a  
 338 more accurate control over drug release from the polymeric carrier. However, it should be  
 339 noted that nearly 80% is released from the total drug content in the case of cl-HyA/100% at  
 340 the end of the seventh hour (**Figure 8A**). On the other hand, using the least amount of  
 341 surfactant (**Figure 8B**) caused only 60% of the active ingredient to dissolve during the  
 342 experiment.

343



344

345 **Figure 8.** Release profiles and Weibull kinetic model-predicted release curves of KP from  
 346 varying degrees cl-HyA-based (A) and CTAB hydrophobized (B) drug carriers.

347 Keeping regard the values of coefficient of determination, ( $R^2$ ) from **Table 1**, the Weibull  
 348 model was the best and the Korsmeyer and Peppas model was the second best model for the  
 349 hydrophobized polymer. While in the case of cl-polymers the Weibull model was the best,  
 350 and first order model is the second best model for describing the release profiles. Although  
 351 the fitting of the Korsmeyer and Peppas equation does not produce a good description of  
 352 dissolution profile as well as the Weibull model, the values of the matching parameters ( $k_m$   
 353 and  $n$ ) also carry important information. Hydrophobized HyA exhibited a release exponent  $n$ ,  
 354 values of 0.60; 0.70 and 0.75, indicating that the drug release from surfactant – polymer



355 system might have followed both drug diffusion and the erosion of matrix in an anomalous  
 356 non-Fickian manner.

357 **Table 1.** Fitting results of the experimental KP release data to different kinetic equations, for  
 358 several formulations

Formulation	First-order		Korsmeyer–Peppas			Weibull		
	$k$ ( $\text{h}^{-1}$ )	$R^2$	$k_m$ ( $\text{h}^{-n}$ )	$n$	$R^2$	$a$	$b$	$R^2$
20% CTAB	0.16	0.9881	524	0.6039	0.9819	0.0003	0.7935	0.9971
50% CTAB	0.10	0.9892	149	0.6975	0.9941	0.0002	0.8210	0.9978
80% CTAB	0.04	0.9907	41	0.7484	0.9989	0.0001	0.7950	0.9995
cl-HyA/50%	0.50	0.9995	1401	0.5539	0.9799	0.0001	0.9913	0.9995
cl-HyA/75%	0.34	0.9977	698	0.6127	0.9783	0.0001	1.0095	0.9977
cl-HyA/100%	0.26	0.9901	1126	0.5497	0.9886	0.0005	0.7950	0.9986

359 Release exponents of the cross-linked carriers are much closer to the limit ( $n = 0.45$ ) which  
 360 indicates the diffusion-controlled Fickian drug release. In addition, the fact that the value of  
 361 the release exponent of the hydrophobized matrix continually decreases as the amount of  
 362 surfactant increases, shows that the contribution of anomalous diffusion is gets stronger.  
 363 Based on the observation of previous parts (change of the zeta potential, turbidity and particle  
 364 size values as a function CTAB amount), it can also be established that the release of KP from  
 365 the carrier faster and rather diffusion controlled when relatively small amount of electrostatic  
 366 adsorbed surfactant molecules are present in the system. As the polymer-ionic surfactant  
 367 interactions lead to changes in polymer structure the dissolution of the drug becomes slower,  
 368 and the release turns to diffusion and erosion controlled way.

#### 369 4. Conclusion

370 Since KP has some disadvantages as of low bioavailability and short half-life the  
 371 formulation of controlled release dosage forms is needed. The results from both rheology and  
 372 conductometric measurements verified the successful synthesis of two types of formulation by  
 373 cross-linking of HyA or surface modification by CTAB. Turbidity, Zeta-potential and particle  
 374 size analysis enabled the determination of optimal CTAB amount. **The concentration-**  
 375 **dependent structure of the HyA-based carrier was clearly confirmed by several rheological**  
 376 **investigations.** The release mechanism of KP from each formulation tested was evaluated in  
 377 light of the first-order, Korsmeyer–Peppas and Weibull kinetic models. The release of KP

378 from the carrier is faster and rather diffusion controlled when relatively small amount of  
379 electrostatic adsorbed **CTAB** are present in the system. As the polymer-ionic surfactant  
380 interactions lead to changes in polymer structure the dissolution of drug becomes slower and  
381 the release turns to diffusion and erosion controlled way. **However, the loading efficiency**  
382 **shows similar values (93-95%) but comparing the controlled drug release studies of our**  
383 **modified-HyA based systems with e.g. alginate-, gelatin- or acrylic polymer-based KP-**  
384 **containing systems we can conclude that after 4-6 h all of the amount of the encapsulated**  
385 **drug was dissolved from the above mentioned composites while in case of our systems (e.g.**  
386 **cl-HyA/100%) after 7 h only 70% but for CTAB/HyA 50% ca. only 40% of the KP content**  
387 **was dissolved. These results support the better applicability of cl-HyA NPs instead of the**  
388 **above mentioned other biocompatible carriers. We presented a potential applications of**  
389 **effective HyA biomaterial-based colloidal controlled drug release systems which contain**  
390 **hydrophobic NSAID KP molecule.**

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# Cross-linked and hydrophobized hyaluronic acid-based **controlled drug** **release systems**

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## Graphical abstract

