Removal of anionic dyes in aqueous solution by flocculation with cellulose ampholytes

HiroYuki Kono*, Ryo Kusumoto

Department of Science and Engineering for Materials, Tomakomai National College of Technology, Nishikioka 443, Tomakomai, Hokkaido 059 1275, Japan

*Corresponding author (H.Kono): E-mail: kono@sem.tomakomai-ct.ac.jp; Tel. and fax: +81 144 67 8036.

Abstract

Cellulose ampholytes (CAs) prepared from biocompatible and biodegradable carboxymethyl cellulose (CMC) could represent an environmentally friendly alternative for the efficient removal of anionic dyes from water effluents. In order to evaluate their potential for this application, a series of CAs with a degree of cationic substitution (DS_C) between 0.26 and 1.08 were prepared from sodium CMC, by cationization with 2,3-epoxypropyltrimethylammonium chloride. The flocculation ability of the CAs against the anionic dye Acid Red 13 (AR13) was found to strongly depend on the DS_C as well as on the pH of the solution. The highest flocculation ability was observed at lower pH and higher DS_C : a color removal as high as 99% was achieved at pH 3 using a CA with the highest DS_C . The kinetic analysis revealed that the flocculation of AR13 can be accurately fitted by a pseudo-second order kinetic model, which thus allows to predict the flocculation behavior of CAs under these conditions. Besides AR13, the ampholytes also showed high flocculation ability against Acid Red 9 and Acid Blue 13. The flocculation behavior of these anionic dyes followed the Langmuir adsorption isotherm model, which revealed the maximum flocculation capacity of a CA for these dyes. Moreover, the analysis of the flocculation isotherm data and the SEM observation of the formed flocs allowed to determine the flocculation mechanism.

Keywords: Cellulose; Amphoteric polymer; Flocculant; Dye removal; Anionic dye.

28 **Nomenclature**

29 CMC, Carboxymethyl cellulose

30 EPTMAC, Epoxypropyltrimethylammonium chloride

31 CAm, Cellulose ampholyte

32 DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid

33

34 **1. Introduction**

35 More than 7×10^5 tons of dyes are used annually in the chemical industry worldwide,
36 about 10–15% of which are discharged into water as effluents, seriously polluting the
37 environment and affecting human as well as aquatic organisms [1, 2]. There are more than
38 ten thousand types of commercially available dyes, most of which are considered toxic [1–3].
39 In addition, most dyes have complex and very stable aromatic structures, and thus tend to
40 accumulate in nature [4–6]. Industrial treatments to remove dyes can involve oxidation [7,
41 8], coagulation [8, 9], flocculation [10, 11], ion-exchange [12], photochemical destruction
42 [13], and other methods. In particular, adsorption has been widely used for the removal of
43 dyes from effluents, and activated carbon is conventionally used for adsorbing dyes from
44 wastewaters. However, the use of activated carbon is limited by its relatively high cost and
45 difficult regeneration [14].

46 Coagulation of dye-containing wastewater has been used for many years either as a
47 pre-treatment or as the main method to treat wastewater due to its low capital cost
48 [15]. Recently, much attention has been devoted to a coagulation-flocculation process for
49 dye removal from water, which employs natural polysaccharides such as sodium alginate
50 [16], carrageenan [17], pectin [18], carboxymethyl cellulose [19], chitosan [20], and
51 carboxymethyl chitin [20]. These compounds are particularly attractive due to their
52 biocompatibility, biodegradability, and anionic properties. However, even though they
53 exhibited promising results, the use of these polysaccharides as anionic flocculation agents
54 is still infrequent. The practical use of anionic polysaccharides as flocculation reagents is
55 limited by their poor solubility in acidic solutions [21, 22]. Chitosan, a cationic polysaccharide

56 abundant in nature, exhibits excellent flocculation properties towards anionic dyes and
57 suspended particles with negatively charged surface. The amino groups of chitosan are
58 protonated and thus charged positively in acidic solutions, resulting in facile dissolution and
59 excellent flocculation performance in acidic solutions [22]. On the other hand, the poor
60 solubility in neutral and alkaline solutions prevent the use of chitosan as flocculant in these
61 conditions [22].

62 Among the naturally occurring polysaccharides, cellulose—which is the most abundant
63 natural polymer on earth—represents one of the most environmentally friendly non-food
64 sources for the production of a wide range of eco-friendly products. The high degree of
65 polymerization and number of potential chemical modifications of cellulose also make it an
66 attractive candidate for flocculation purposes [23, 24]. For example, sodium carboxymethyl
67 cellulose (CMC), a well known water-soluble cellulose derivative with properties
68 characteristic of an anionic polymer, has shown promising results as an anionic flocculation
69 agent, despite the poor solubility in acidic solutions that limits its practical application
70 as a flocculation reagent in these conditions [19]. In addition, cationized cellulose, produced by
71 homogeneous cationization with 2,3-epoxypropyltrimethylammonium chloride
72 (EPTMAC), was reported to exhibit good cationic flocculation ability toward kaolin
73 suspensions [25]. However, as cellulose is highly crystallized by a strong network of inter-
74 and intra-molecular hydrogen bonds, its dissolution requires a time-consuming processing
75 with unpopular solvents such as aqueous sodium hydroxide/urea solution, lithium
76 chloride/dimethyl sulfoxide, tetra-n-butylammonium fluoride/dimethyl sulfoxide, and specific
77 ionic liquids, and/or preswelling by sequential solvent change and heating [26]. In our
78 previous work, cellulose ampholytes (CAs) containing both cationic quaternary ammonium
79 sodium salt and anionic carboxymethyl groups were prepared from homogeneous
80 etherification of CMC dissolved in aqueous solution in the presence of EPTMAC as cationic
81 reagent. The starting CMC material is relatively cheap and easily dissolved in NaOH
82 solution, and its unsubstituted hydroxyls can easily react with EPTMAC to form cationic
83 groups. Thanks to these cationic groups, the obtained CAs could easily dissolve in

solutions within a wide pH range and exhibited excellent flocculation performance toward suspended kaolin particles [27]. Similarly, it was reported that several polysaccharide-based amphoteric flocculants, including chitosan [28], amylopectin [29], and starch [30], showed enhanced solubility and good flocculation performance.

In this study, a series of CAm having a different degree of cationic substitution (DS_C) was prepared from CMC with a degree of anionic substitution (DS_A) of 0.60 (Fig. 1(A)), and the effects of several factors on the flocculation ability of the prepared CAm against the Acid Red 13 (AR13) anionic dye in aqueous solution were investigated in detail. In addition, in order to determine the mechanism of interaction between CAm and the anionic dyes during flocculation, we investigated the flocculation behavior of CAm towards Acid Red 9 (AR9), AR13, and Acid Blue 92 (AB92) anionic dyes, which contain 1, 2, or 3 anionic sulfone groups per molecule, respectively (Fig. 1(B)).

(Fig.1)

2. Materials and methods

2.1. Materials

CMC ($DS_A = 0.60$, molecular weight (M_w) = 2.6×10^5 g mol⁻¹) was kindly supplied from Dai-ichi Kogyo Seiyaku Co. (Japan). EPTMAC was purchased from Sigma-Aldrich (USA). AR9, AR13, and AB92 were all purchased from Tokyo Chemical Industry Co. Ltd. (Japan). The molecular weights of AR9, AR13, and AB92 were 400.4, 502.4, and 695.6 g mol⁻¹, respectively. Other chemicals were chemically pure grade and all solutions were prepared with pure water.

2.2 Synthesis of CAm

A series of four CAm (labeled **1–4**) were prepared from CMC with EPTMAC according to the method previously reported [27]. Briefly, CMC (1.0 g) was dissolved in 1.5 mmol L⁻¹ NaOH solution at 277 K, and then EPTMAC (1.4 g) was added dropwise over a period of 20 min. After precipitation with acetone, the obtained precipitates were washed with a 1:1

111 mixture of acetone/distilled water to reach neutral pH, dried under vacuum, and then milled
112 and filtered through a 40-mesh sieve to obtain CAM1. A similar method was used to prepare
113 CAMs 2–4, by adapting the feed amount of EPTMAC, which was set to 3.5 g, 4.9 g, and 7.0
114 g for CAMs 2, 3, and 4, respectively. All CAMs were stored in a desiccator under vacuum
115 until ready for use.

116

117 **2.3. ¹³C NMR spectroscopy**

118 NMR spectra of the CAMs were measured at 353 K in deuterium oxide containing 0.1%
119 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal standard using an AV III
120 500 MHz spectrometer equipped with a 5mm BBO probe (Bruker BioSpin GmbH,
121 Germany). The quantitative ¹³C NMR spectra were obtained by the inverse-gated ¹H
122 decoupling method [31] with a flip angle for ¹³C excitation pulse of 30° and a pulse
123 repetition time of 45 s [32]. The ¹³C chemical shifts were calibrated by setting the methyl
124 peak of DSS to 0 ppm.

125

126 **2.4. Flocculation of anionic dyes**

127 Three kinds of anionic dyes, whose structures are shown in Fig. 1(B), were used for the
128 flocculation studies. A buffer solution (10 mL) containing each dye in a concentration range
129 of 50–1600 mg L⁻¹ was poured in a glass vial, and the CAM was added to the dye solution.
130 The mixture was immediately placed in a shaker at 120 rpm and 298 K for 1 h, after which
131 the suspension was left to settle for 5 min. The supernatant of the suspension was filtered
132 by using a Nylon membrane filter with a pore size of 0.45 μm (Rephile Bioscience, Ltd.,
133 USA). The dye concentration of the filtrate was determined by an Epoch 96 well micro-
134 volume spectrophotometer (BioTek Instruments, Inc., USA) with a 96-wells microplate,
135 using an earlier prepared calibration curve at 492 nm for AR9, 504 nm for AR13, and 572
136 nm for AB92. The percent removal of each dye and the flocculation ability (q_t) of each CAM
137 at contact time t were calculated using Eqs. (1) and (2):

$$138 \text{ (Dye removal)\%} = (C_0 - C) / C_0 \times 100 \quad (1)$$

$$q_t = (C_0 - C) \times V/m \quad (2)$$

where C_0 (mg L^{-1}) is the initial concentration of dye solution, C (mg L^{-1}) is the concentration of dye solution at contact time t , V (L) is the volume of dye solution, and m (g) is the mass of CAm. 20 mM citric acid-sodium citrate (pH 3–6), 20 mM NaH_2PO_4 - Na_2HPO_4 (pH 7–8), 20 mM Tris-HCl (pH 9.0), and 20 mM NaHCO_3 -NaOH (pH 10) were used as buffer solutions for the flocculation media.

2.5. SEM measurements

Scanning electron microscopy (SEM) images of the samples were obtained using a JSM 6330F scanning electron microscope (JEOL Ltd., Japan), using a potential of 1 kV and Au vapor deposition to prepare the sample surface.

3. Results and discussion

3.1. Structural characterization of CAm

The quantitative ^{13}C NMR spectra of CAm 1–4 in D_2O are shown in Fig. 2, which also highlights previous assignments of ^{13}C resonances [27]. As the EPTMAC feed amount used in the preparation of the CAm was increased, the intensity of the 57 ppm peak corresponding to the methyl carbon in the cationic groups was enhanced, while the signal of the carbonyl carbon in the anionic $-\text{COO}^-\text{Na}^+$ groups at 180 ppm hardly changed. The integrals of the ^{13}C signals for the methyl and carbonyl carbons ($I_{\text{C=O}}$ and I_{CH_3} , respectively) and for the anomeric carbon at 105 ppm (I_{C1}) in each spectrum can be used to determine the DS_C and DS_A according to Eqs. (3) and (4):

$$\text{DS}_\text{A} = I_{\text{C=O}}/I_{\text{C1}} \quad (3)$$

$$\text{DS}_\text{C} = I_{\text{CH}_3}/(3 \times I_{\text{C1}}) \quad (4)$$

The DS_C and DS_A values can then be used to calculate the yield obtained for each CAm through Eqs. (5) and (6):

$$\text{Yield}(\%) = \text{Yield (g)} / \text{Mw}_{\text{AGU}} (\text{g mol}^{-1}) \times 100 / 4.6 \times 10^{-3} (\text{mol}) \quad (5)$$

where Mw_{AGU} is the average molecular weight of the anhydroglucose unit for each

CAM, which can be calculated through Eq. (6):

$$\text{Mw}_{\text{AGU}} (\text{g mol}^{-1}) = 162 + 151.5 \times \text{DS}_{\text{C}} + 80 \times \text{DS}_{\text{A}} \quad (6)$$

Table 1 summarizes the DS_{C} , DS_{A} , and the yield of each CAM. The DS_{C} values for CAMs

1–4 were 0.26, 0.66, 0.83, and 1.08, respectively, while the DS_{A} of all CAMs was 0.60,

indicating that no dissociation of anionic groups or ether exchange between anionic and

cationic groups had occurred during the preparation of the CAMs. The yields were in range

of 88–92%. These data are in good agreement with values previously reported [27].

(Fig. 2 and Table 1)

3.2. Effects of external parameters on AR13 flocculation

3.2.1. Effect of solution pH

Among the external parameters which can affect the flocculation efficiency, the pH of the flocculation system is the most important one [27–30]. In addition, the charge balance between cationic and anionic groups of the amphoteric flocculants may also have great influence on the flocculation efficiency [27–30]. Therefore, we assessed the effects of these parameters on the flocculation ability of CAMs toward AR13.

Fig. 3(A) shows the flocculation ability (q) for each CAM, defined as the amount of flocs per gram of CAM, at various pH ranging between 3 and 10. All CAMs showed a maximum flocculation ability at pH 3. The flocculation ability was sharply reduced when the pH was increased from 4 to 5, and then slowly decreased with further pH increases from 5 to 10. In addition, the flocculation ability in all pH solutions follows the order CAM 1 < CAM 2 < CAM 3 < CAM 4. Taking into account that AR13 is present in anionic form in the pH range investigated, the attraction between each CAM and AR13 is the dominant force that generates the dye flocculation, and the above result can be attributed to the different structure of the CAMs studied here, which in turn depends on the solution pH.

191 The pKa of the carboxymethyl groups of CMC was reported to be around 4.3 [33], and
192 the same value is also attributed to the pKa of the anionic groups within the CAM chains.
193 When the pH is set to lower than 4, the negative charges on the surface of the CAMs
194 vanish due to the protonation of their carboxylate groups, and thus each CAM acts as a
195 cationic polymer with a specific DS_C value. The anionic dyes can then diffuse to the
196 positively charged surface of the CAM chains by electrostatic attraction forces, so that their
197 flocculation occurs via a charge neutralization mechanism. Therefore, the maximum
198 flocculation ability of all CAMs could be achieved at pH 3. On the other hand, when the pH
199 of the solution is higher than 5, the surface of CAMs bears both positive and negative
200 charges, according to the DS_C and DS_A values of the polymer. In these conditions,
201 intermolecular and intramolecular charge neutralization is considered to occur between the
202 cationic groups and anionic groups of the CAM chains, suggesting that above pH 5, the
203 effective cationic charge of each CAM should be considerably lower than the DS_C of the
204 polymer. Therefore, the drastic decrease in flocculation ability of the CAMs with increasing
205 solution pH is due to the lower cationic charge of the polymer surface, as well as to the
206 electrostatic repulsion between the anionic charge of the polymer surface and the AR13
207 molecules. These results thus denote that, in order to flocculate the anionic dye, a
208 combination of low pH of the flocculation medium and a high DS_C of the CAM sample is
209 preferable.

210 The effect of aggregation is considered in the flocculation from AR13 with CAMs. The
211 cationic groups of CAMs bind to the anionic sites in AR13. Charge neutralization between
212 an AR13 molecule and CAM chains enhanced the hydrophobicity of the formed polymer-
213 dye complexes. As a result, hydrophobic interactions occur between the polymer-dye
214 complex, which results in the aggregation of the complex and the formation of macro floc.
215 In addition, electrostatic interactions between the unneutralized cationic group of the
216 polymer-dye complex and unneutralized anionic group of the other complex is considered
217 to prompt the aggregation.

(Fig. 3)

3.2.2. Effect of temperature

The flocculation of AR13 by CAmS 1–4 was investigated in the temperature range of 298–333 K, and the results are shown in Fig. 3(B). The flocculation ability of all CAmS stays almost constant in the temperature range investigated, indicating that the temperature of the dye solution has no influence on the floc formation of AR13 with CAmS. If the flocculation was affected by the temperature, then its mechanism would also involve physical adsorption, in addition to the electrostatic adsorption between the dye and CAm chains, because physical adsorption processes controlled by interactions such as Van der Waals forces generally exhibit a strong temperature dependence [35]. Therefore, this finding indicates that the floc formation can be described as a homogeneous adsorption process induced by the electrostatic interaction between the anionic dye and the cationic groups of the CAm chains.

3.2.3. Effect of the flocculant dose

To evaluate the effect of the CAm dose on the flocculation of AR13, CAmS 1–4 with a variable flocculant dose (50–1600 mg L⁻¹) were exposed to a fixed initial dye concentration of AR13. The results in Fig. 3(C) show that the extent of color removal increased with increasing flocculant dose up to an optimal value, followed by a decreasing trend with further increases in dose. These curves are typical of flocculant systems controlled by a charge neutralization mechanism [28–30, 35]. In particular, when the amount of cationic charge was sufficient for the complete neutralization of the anionic charge, the color removal reached the maximum efficiency. Excess of cationic charge following overdosing of the flocculant induced a dispersion re-stabilization phenomenon and a subsequent decrease in the efficiency of the flocculation process [35].

244 As shown in Fig.3(C), the DS_C had strong influence on the optimal dose of
245 flocculant:for CAmS with high DS_C values, the optimal color removal could be reached at
246 lower flocculant doses. The optimum doses of CAm 1–4were250, 400, 800, and 1400 mg L⁻¹,
247 respectively. The decrease in the optimal flocculant dose is due to the increase in the
248 number of available sorption sites in the CAm chains. In addition, the maximum color
249 removal of AR13 at the optimal dose for each CAm decreased with decreasing DS_C of the
250 polymer employed: the maximum color removal efficiency was 99.3% for CAm 4, 97.9% for
251 CAm 3, 90.6% for CAm 2, and 78.2% for CAm 1 at the optimal flocculant dosages.This
252 suggests that ahigher DS_C could help to enhancethe hydrophilicity of CAm in water, leading
253 to increased dye binding due to higher accessibility of cationic groups along CAm chains
254 [35].

255 From these observations, the most effective flocculant was determined to be CAm 4,
256 and the optimal conditions for AR13 removal by CAm 4were pH 3 and a flocculant dosage
257 of 250 mg L⁻¹. Under these optimal conditions, the AR13 removal efficiency was 99.3 %.
258 This efficiency is equal to or greater than that achieved using other coagulant/flocculation
259 techniques for AR dye removal. For example, polyammonium chloride (PAC),which is a
260 well-known inorganic polymer flocculant,exhibitsexcellent performance in the removal of
261 various dyestuffs, including AR119, and the optimal pH, coagulation dosage, and initial
262 AR119 concentration were 3.8, 57 mg L⁻¹, and 140 mgL⁻¹, respectively. Under these
263 optimal conditions, the dye removal efficiency is 95.25 % [36]. Therefore, although the
264 optimal conditions for CAm 4 differ from those for PAC, the CAmS with high DS_C values are
265 effective flocculants for the removal of anionic dyes.

266

267 **3.3. Kineticanalysis**

268 The contact time between flocculants and dye molecules is another important
269 parameter for the flocculation processes because it can provide information onthe
270 flocculation kineticsof the dyes for a given initial concentration of flocculant [28–30].
271 Therefore, the effect of contact time on the flocculation ability of CAmS 2–4toward

AR13 was investigated at pH 7, and the data are shown in Fig. 4(A). The flocculation behavior of AR13 by each CAM can be divided into three regions: the flocculation ability rapidly increased in the first 10 min, after which it gradually increased until equilibrium was reached at a contact time of 25 min, and then remained constant. The behavior in the first region is due to the rapid diffusion of the anionic dye molecules to the cationic groups on the surface of CAM chains and the subsequent charge neutralization. The second region, with a slower increase in flocculation ability, is due to the decrease of the effective cationic charge on the CAM surface. After 25 min into the third region, there was almost no further increase in adsorption of AR13 onto the CAM, indicating that the equilibrium time for the floc formation is about 25 min under the flocculation conditions investigated here.

Several kinetic models have been previously used to describe the kinetics of dye flocculation by polysaccharide-based flocculants [37]. Among these models, a pseudo-first order [38] and a pseudo second order kinetic model [39] were used to fit the experimental data for the flocculation of AR13 by CAMs. The pseudo-first order and pseudo-second order kinetic models are expressed by the following formulas:

$$\ln(q_e - q_t) = \ln(q_e) - k_1 t \quad (7) \text{ and}$$

$$t/q_t = k_2^{-1} q_e^{-2} + q_e^{-1} t \quad (8)$$

respectively, where k_1 and k_2 are the rate constants for the pseudo-first order and pseudo-second order kinetic models, while q_e and q_t are the flocculation abilities of each CAM at equilibrium and contact time t , respectively. The slopes and intercepts of $\ln(q_e - q_t)$ versus t plots were used to determine the pseudo-first order rate constant k_1 and the theoretical flocculation ability at equilibrium, $q_{e,cal}$. If the pseudo-second order model was applicable, the slopes and intercepts of plots of t/q_t versus t were used to determine the pseudo-second order rate constant k_2 and the corresponding theoretical flocculation ability at equilibrium $q_{e,cal}$ (Fig. 3(C)). The equilibrium time in the figure was set to 60 min, and for each CAM q_e was set to the q value at $t = 60$ min. The kinetic parameters for the two kinetic models determined from the curve-fitting plots are presented in Table 2. The correlation coefficients

(R^2) for the pseudo-second order kinetic model are in the range of 0.998–0.999, considerably higher than those of the pseudo-first order model ($R^2=0.803$ –0.923). Moreover, it was found that the theoretical $q_{e,cal}$ values determined by the pseudo-second order model were more consistent with the experimentally determined flocculation ability at equilibrium $q_{e,exp}$, compared with the $q_{e,cal}$ determined by the pseudo-first order kinetic model. This suggests that the pseudo-second order adsorption mechanism was predominant for the adsorption of the AR13 onto the CAmS. A similar kinetic behavior has been reported for the electrostatic adsorption of ionic dyes onto polysaccharide-based flocculants [29, 30, 35, 37]. Therefore, it is possible to predict the flocculation behavior of AR13 in the presence of CAmS over the entire range of contact time by using the rate constant k_2 determined for the pseudo-second order kinetic model.

(Fig. 4 and Table 2)

3.4. Flocculation isotherms

Fig. 5 shows the effect of the initial concentration (C_0) of AR9, AR13, and AB92 dyes on the flocculation ability at equilibrium (q_e) of Cam 3 at pH 3 and 7. For all cationic dyes, high q_e values were observed with increasing C_0 , and saturation levels were gradually achieved at $C_0 = 300 \text{ mg L}^{-1}$. In addition, the saturated q_e value at pH 7 was considerably lower than that at pH 3, suggesting that all cationic dyes were flocculated with the CAmS via the charge neutralization mechanism.

Adsorption phenomena are usually described through isotherms, i.e., plots of the amount of adsorbate on the adsorbent as a function of pressure (in the case of a gas) or concentration (in the case of a liquid at constant temperature). The adsorption isotherm is important for identifying the interaction of the adsorbates on the adsorbents and for optimizing the use of adsorbents. Among a number of different isotherms, the Langmuir and Freundlich models are the most popular ones to describe the adsorption of a dye onto a polymer surface [40, 41], and were applied here to analyze the flocculation of cationic dyes AR9, AR13, and AB92 by the CAmS.

The basic assumption of the Langmuir model is that adsorption occurs at specific homogeneous sites on the adsorbent. A further assumption is that once an adsorbate occupies a site, no further adsorption can take place at the same site [41]. The linear form of the Langmuir equation can be expressed by Eq. (9):

$$q_e^{-1} = Q_0^{-1} + (bQ_0C_e)^{-1} \quad (9)$$

where q_e is the amount of adsorbate on the adsorbent at equilibrium (mg g^{-1}), Q_0 is the maximum monolayer coverage capacity (mg g^{-1}), b is the Langmuir isotherm constant (L mg^{-1}), and C_e is the equilibrium concentration of adsorbate (mg L^{-1}). Moreover, a dimensionless constant commonly known as separation factor (R_L), as defined by Weber and Chakravarti [42], can be defined as:

$$R_L = (1 + bC_0)^{-1} \quad (10)$$

Based on the R_L values, the adsorption process can be categorized as unfavorable ($R_L > 1$), linear ($R_L = 1$), favorable ($0 < R_L < 1$), or irreversible ($R_L = 0$). On the other hand, the linearized form of the Freundlich isotherm equation is given by Eq. (11) [41]:

$$\ln q_e = \ln K_F + n^{-1} \ln C_e \quad (11)$$

where K_F is the Freundlich isotherm constant, which is related to the adsorption capacity, and the dimensionless constant n^{-1} is an empirical parameter, which gives valuable information on the isotherm shape. Based on the n^{-1} values, the adsorption process may be classified as irreversible ($n^{-1} = 0$), favorable ($0 < n^{-1} < 1$), or unfavorable ($n^{-1} > 1$).

Based on the data in Fig. 5, linear plots corresponding to the Langmuir and Freundlich isotherm models are presented in Fig. 6, and the linearized isotherm coefficients of these models, as determined from the plots, are summarized in Table 3. As observed in Fig. 6 and Table 3, the dimensionless constants R_L obtained from the Langmuir model are in the 0.115–0.255 range, indicating favorable adsorption of all anionic dyes onto CAmS. Additionally, in the case of the Freundlich model, the empirical constants n^{-1} lie in the range of 0.655–0.908, also indicating favorable adsorption of the dyes onto CAmS. However, the correlation coefficients R^2 for the Langmuir model are in range of 0.996–0.999, while the Freundlich model gives lower values, between 0.942 and 0.991. Additionally, as shown

in Table 3, the maximum monolayer coverage capacities (Q_0) of CAM 4 for AR9, AR13, and AB92 at pH 3 are 558 mg g⁻¹, 450 mg g⁻¹, and 457 mg g⁻¹, and those at pH 7 are 270 mg g⁻¹, 195 mg g⁻¹, and 140 mg g⁻¹, respectively. These data are in good agreement with the equilibrium flocculation ability of CAM 4 at the initial concentration of 600 mg L⁻¹ shown in Fig. 5. They also imply that the adsorption of all anionic dyes onto CAM follows a Langmuir-type adsorption mechanism. The Langmuir constant b , which describes the desorption process of an anionic dye adsorbed on a CAM is of the order of 10⁻²–10⁻³, indicating that the flocs formed from anionic dyes by the CAM are very stable in solution.

The Langmuir adsorption isotherm model is based on the following assumptions, although some exceptions have been reported [43]:

- The adsorbent surface is uniform, i.e., all adsorption sites are equivalent.
- The adsorbed molecules do not interact with each other.
- All molecules are adsorbed by the same mechanism.
- At the maximum adsorption, only a monolayer is formed. In other words, the adsorbate molecules do not deposit on other molecules previously adsorbed on the adsorbent.

Therefore, based on the fact that our data fit well the Langmuir model, it can be concluded that the flocculation of dye molecules follows a homogeneous mechanism based on the charge neutralization between dye molecules and the cationic surface charge of CAM. This suggests that hydrophobic interactions between the dye molecules, for example π – π stacking interactions [44] between aromatic rings of cationic dyes, are small enough to be negligible, compared with the electrostatic interaction between the cationic dye and anionic CAM surface. If other interactions besides the electrostatic ones played a role in the flocculation of anionic dye, then the flocculation isotherm would be closer to the Freundlich adsorption isotherm model, which is generally employed to describe heterogeneous adsorption systems [40].

(Figs. 5 and 6, and Table 3)

3.5. SEM analysis

381 Finally, the flocculation of AR9, AR13, and AB92 by CAm 4 was performed at pH3 (Fig.
382 7A), and the formed floc was freeze-dried and then observed by SEM (Fig.
383 7B). The morphologies of the flocs formed from AR9 were completely different from those
384 formed from AR13 and AB92. The flocs flocculated from AR9 contained small linear
385 particles, which aggregated with each other to form the large flocs. On the other hand, flocs
386 formed from AR13 and AB92 exhibited well-developed porous structures, with pore sizes of
387 about 50–200 μm and 10–60 μm for AR13 and AB92, respectively. Such porous network
388 structures have been frequently observed in the morphology of crosslinked polymers such
389 as hydrogels: for example, hydrogels prepared from cellulose [45], carboxymethyl cellulose
390 [33], chitosan [46], chitin [47], and guar gum [48], crosslinked by bifunctional crosslinking
391 agents, showed similar porous morphologies. As described above, all polymer surfaces are
392 positively charged at pH 3 due to the protonation of anionic carboxymethyl groups, and
393 thus the floc formation takes place through the charge neutralization mechanism between
394 the anionic dye and the positively charged polymer surface. Therefore, it can be suggested
395 that the morphological differences between the flocs formed from AR9, AR13, and AB92
396 reflect the structural differences between AR9, AR13, and AB13 molecules, each containing
397 1, 2, and 3 anionic sulfone groups, respectively.

398 In the case of flocculation of AR9 at pH 3, the AR9 molecule diffuses to neutralize the
399 positively charged surface of a CAm chain. As the charge neutralization progresses, the
400 polymer surface loses the cationic character, while its hydrophobicity sharply increases due
401 to the surface being covered by AR9. Finally, as the polymer surface becomes neutral,
402 polymer deposition occurs in the solution, and the hydrophobic interaction between the
403 polymer chains leads the polymers to aggregate to each other to form the macroflocs,
404 visible in the SEM photograph in Fig 7. Similarly to the floc formation from AR9, floc
405 formation from AR13 and AB92 also involved the sulfone group of each dye
406 molecule interacting with a cationic group on the CAm surface and neutralizing the
407 corresponding charge. The remaining unreacted sulfone group(s) of the AR13 or
408 AB92 molecules can also readily react with the other cationic groups on the polymer

surface. In particular, the sulfone group(s) of the dye attached on the CAm chain could interact with the same polymer chain, resulting in the formation of intramolecular crosslinks in the CAm mediated by the dye molecule. On the other hand, if the sulfone group(s) of the anionic dye interact with the other CAm chains, intermolecular crosslinking between CAm chains mediated by dye molecules can occur, resulting in a highly interconnected network structure. The morphology of flocs formed from AR13 and AB92 (Fig. 7) suggests that the porous structures were formed through the intra- and intermolecular crosslinking of CAm chains via the anionic dyes. In addition, it can be argued that the different pore sizes observed for the network structure of flocs formed from AR13 and from AB92 result from the different number of anionic sulfonic groups per molecule: AR13 has two sulfonic groups able to connect two chains of CAm via intermolecular crosslinking, while AB13 can crosslink up to three CAm chains through the three sulfonic groups in its structure. With respect to the proportion of intra- versus intermolecular crosslinking in the flocs formed from AR13 and AB92, the rather diluted initial concentration of CAm is considered to favor the formation of intramolecular rather than intermolecular crosslinking, although their relative exact proportions could not be determined only from the morphological observation of the flocs.

(Fig. 7)

4. Conclusions

In order to assess the potential performance and suitability of cellulose ampholytes in treatments to remove dyes from water effluents, in this study we investigated the flocculation ability of various CAmS obtained from CMC towards anionic dyes. The main findings were the following:

1. The flocculation ability strongly depends on the DS_C of the CAm used as a flocculant and on the pH of the flocculation medium (Fig. 3). In particular, a high flocculation efficiency can be achieved by flocculation at lower pH and using CAmS having high DS_C . The maximum color removal efficiency measured was 99.2 % for AR13 at pH 3.

436 2. Flocculation of AR13 by CAm proceeds by a pseudo-second order kinetic
437 mechanism (Fig. 4).
438 3. The flocculation of AR9, AR13, and AB13 followed the Langmuir, rather than
439 Freundlich, adsorption isotherm model (Fig. 6), suggesting that the flocculation of
440 these cationic dyes was predominantly controlled by a charge neutralization
441 mechanism, and that physical (such as hydrophobic) interactions between the dye
442 molecules were negligible.

443 Based on these findings, it can be concluded that the CAm obtained in this study had
444 high flocculation ability towards anionic dyes and may be suitable flocculants to readily
445 remove anionic dyes in solution. In addition, since the starting CMC material is nontoxic and
446 biocompatible [49], these CAm are also environmentally and biologically safe, thus they
447 represent strong candidates as synthetic polymer flocculants for dye removal from effluents.
448

449 **Acknowledgements**

450 This work was partially supported by Grants-in-Aid for Scientific Research C-25410134
451 from the Japan Society for Promotion of Science (JSPS), and by Grants-in-aid for the
452 Adaptable and Seamless Technology Transfer Program through Target-driven R&D
453 AS262Z01980M from the Japan Science and Technology Agency (JST).
454

455 **References**

- 456 [1] T.K. Sen, S. Afroze, H.M. Ang, Equilibrium, kinetics and mechanism of removal of
457 methylene blue from aqueous solution by adsorption onto pine cone biomass of *Pinus*
458 *radiata*, *Water Air Soil Poll.* 218 (2011) 499–515.
459 [2] M.T. Yagub, T.K. Sen, H.M. Ang, Equilibrium, kinetics, and thermodynamics of
460 methylene blue adsorption by pine tree leaves, *Water Air Soil Poll.* 223 (2012) 5267–
461 5282.
462 [3] M.T. Yagub, T.K. Sen, S. Afroze, H.M. Ang, Dye and its removal from aqueous solution
463 by adsorption: A review, *Adv. Colloid Interface Sci.* 209 (2014) 172–184.
464 [4] Y. Fu, T. Viraraghavan, Fungal decolorization of dye waste waters: a review. *Biores.*
465 *Technol.* 79 (2001) 251–262.

- 466 [5] T. Lazar, Color chemistry: synthesis, properties, and applications of organic dyes and
467 pigments, 3rd revised edition. Color Res. Appl. 30 (2005) 313–314.
- 468 [6] K. Kadivelu, M. Kavipriya, C. Kathika, M. Radhika, N. Vennilamani, S. Patabhi,
469 Utilization of various agricultural wastes for activated carbon preparation and
470 application for the removal of dyes and metal ions from aqueous solutions, Biores.
471 Technol. 87 (2003) 129–132.
- 472 [7] M. Punzi, A. Anbalagan, R. A. Börner, B.-M. Svensson, M. Jonstrup, B. Mattiasson,
473 Degradation of a textile azo dye using biological treatment followed by photo-Fenton
474 oxidation: Evaluation of toxicity and microbial community structure, Chem. Eng. J. 270
475 (2015) 290–299.
- 476 [8] N. Azbar, T. Yonar, K. Kestioglu, Comparison of various advanced oxidation processes
477 and chemical treatment methods for COD and color removal from a polyester and
478 acetate fiber dyeing effluent, Chemosphere 55 (2004) 35–43.
- 479 [9] S.V. Mohan, P. Sailaja, M. Srimurali, J. Kathikeyan, Color removal of monoazo acid dye
480 from aqueous solution by adsorption and chemical coagulation, Environ. Eng. Policy 1
481 (1998) 149–154.
- 482 [10] A.K. Verma, R.R. Dash, P. Bhunia, A review on chemical coagulation/flocculation
483 technologies for removal of colour from textile wastewaters, J. Environ. Manage. 93
484 (2012) 154–168.
- 485 [11] Y.-Y. Lau, Y.-S. Wong, T.-T. Teng, N. Morad, M. Rafatullah, S.-A. Ong, Coagulation-
486 flocculation of azo dye Acid Orange 7 with green refined laterite soil, Chem. Eng. J.
487 246 (2014) 383–390.
- 488 [12] V.V. Sethuraman, B.C. Raymahashay, Color removal by clays. Kinetic study of
489 adsorption of cationic and anionic dyes, Environ. Sci. Technol. 9 (1975) 1139–1140.
- 490 [13] C. Galindo, P. Jacques, A. Kalt, Photochemical and photocatalytic degradation of an
491 indigoid dye: a case study of acid blue 74 (AB74), J. Photochem. Photobiol. A Chem.
492 141 (2001) 47–56.
- 493 [14] P.K. Malik, Dye removal from wastewater using activated carbon developed from
494 sawdust: adsorption equilibrium and kinetics, J. Hazard. Mater. 113 (2004) 81–88.
- 495 [15] A.Y. Zahrim, C. Tizaoui, N. Hilal, Coagulation with polymers for nanofiltration pre-
496 treatment of highly concentrated dyes: A review, Desalination 266 (2011) 1–16.
- 497 [16] V. Rocher, J.-M. Siaugue, V. Cabuil, A. Bee, Removal of organic dyes by magnetic
498 alginate beads, Water Res. 42 (2008) 1290–1298.
- 499 [17] G.R. Mahdavinia, F. Bazmizynabad, B. Seyyedi, *kappa*-Carrageenan beads as new
500 adsorbent to remove crystal violet dye from water: adsorption kinetics and isotherm,
501 Desalin. Water Treat. 53 (2015) 2529–2539.

- 502 [18] E.A. Pavan, A.C. Mazzonato, Y. Gushikem, Removal of methylene blue dye from
503 aqueous solutions by adsorption using yellow passion fruit peel as adsorbent, *Biores.*
504 *Technol.* 99 (2008) 3162–3165.
- 505 [19] M.F.A. Taleb, H.L.A. El-Mohdy, H.A.A. El-Rehim, Radiation preparation of PVA/CMC
506 copolymers and their application in removal of dyes, *J. Hazard. Mater.* 168 (2009) 68–
507 75.
- 508 [20] A. Bhatnagar, M. Sillanpää, Applications of chitin- and chitosan-derivatives for the
509 detoxification of water and wastewater — A short review, *Adv. Colloid Interface Sci.*
510 152 (2009) 26–38.
- 511 [21] R.S. Blackburn, Natural polysaccharides and their Interactions with dye molecules:
512 Applications in effluent treatment, *Environ. Sci. Technol.* 38 (2004) 4905–4909.
- 513 [22] G. Crini, P.-M. Badot, Application of chitosan, a natural aminopolysaccharide, for dye
514 removal from aqueous solutions by adsorption processes using batch studies: A
515 review of recent literature, *Prog. Polym. Sci.* 33 (2008) 399–447.
- 516 [23] G. Annadurai, R.-S. Juang, D.-J. Lee, Use of cellulose-based wastes for adsorption of
517 dyes from aqueous solutions, *J. Hazard. Mater.* 92 (2002) 263–274.
- 518 [24] V.K. Gupta, Suhas, Application of low-cost adsorbents for dye removal – A review, *J.*
519 *Environ. Manage.* 90 (2009) 2313–2342.
- 520 [25] L. Yan, H. Tao, P.R. Bangal, Synthesis and flocculation behavior of cationic cellulose
521 prepared in a NaOH/urea aqueous solution, *Clean* 37 (2009) 39–44.
- 522 [26] S. Sen, J.D. Martin, D.S. Argyropoulos, Review of cellulose non-derivatizing solvent
523 interactions with emphasis on activity in inorganic molten salt hydrates, *Sustainable*
524 *Chem. Eng.* 1 (2013) 858–870.
- 525 [27] H. Kono, R. Kusumoto, Preparation, structural characterization, and flocculation ability
526 of amphoteric cellulose, *React. Funct. Polym.* 82 (2014) 111–119.
- 527 [28] Z. Yang, Y. Shang, Y. Lu, Y. Chen, X. Huang, A. Chen, Y. Jiang, W. Gu, X. Qian, H.
528 Yang, R. Cheng, Flocculation properties of biodegradable amphoteric chitosan-based
529 flocculants, *Chem. Eng. J.* 172 (2011) 287–295.
- 530 [29] R.P. Singh, S. Pal, V.K. Rana, S. Ghorai, Amphoteric amylopectin: a novel polymeric
531 flocculant, *Carbohydr. Polym.* 91 (2013) 294–299.
- 532 [30] Q. Lin, S. Qian, C. Li, H. Pan, Z. Wu, G. Liu, Synthesis, flocculation and adsorption
533 performance of amphoteric starch, *Carbohydr. Polym.* 90 (2012) 275–283.
- 534 [31] Z. Zhou, R. Kümmerle, X. Qiu, D. Redwine, R. Cong, A. Taha, D. Baugh, B. Winniford,
535 A new decoupling method for accurate quantification of polyethylene copolymer
536 composition and triad sequence distribution with ^{13}C NMR, *J. Magn. Reson.* 187
537 (2007) 225–233.

- 538 [32] H. Kono, H. Hashimoto, Y. Shimizu, NMR characterization of cellulose acetate:
539 Chemical shift assignments, substituent effects, and chemical shift additivity,
540 Carbohydr. Polym. 118 (2015) 91–100.
- 541 [33] H. Kono, Characterization and properties of carboxymethyl cellulose hydrogels
542 crosslinked by polyethylene glycol, Carbohydr. Polym. 106 (2014) 84–93.
- 543 [34] A.Y. Zahrim, C. Tizaoui, N. Hilal, Evaluation of several commercial synthetic polymers
544 as flocculant aids for removal of highly concentrated C.I. Acid Black 210 dye, J. Hazard.
545 Mater. 182 (2010) 624–630.
- 546 [35] L. Wang, A. Wang, Adsorption properties of congo red from aqueous solution onto *N*,
547 *O*-carboxymethyl-chitosan, Biores. Technol. 99 (2008) 1403–1408.
- 548 [36] S.S. Moghaddam, M.R.A. Moghaddam, M. Arami, Response surface optimization of
549 acid red 119 dye from simulated wastewater using Al based waterworks sludge and
550 polyaluminium chloride as coagulant, J. Environ. Manage. 92 (2011) 1284–1291.
- 551 [37] E. Guibal, J. Roussy, Coagulation and flocculation of dye-containing solutions using a
552 biopolymer (Chitosan), React. Funct. Polym. 67 (2007) 33–42.
- 553 [38] Y.S. Ho, G. McKay, Sorption of die from aqueous solution by peat, Chem. Eng. J. 70
554 (1998) 115–124.
- 555 [39] Y.S. Ho, G. McKay, Pseudo-second order model for sorption processes, Process
556 Biochem. 34 (1999) 451–465.
- 557 [40] G. Crini, Kinetic and equilibrium studies on the removal of cationic dyes from aqueous
558 solution by adsorption onto a cyclodextrin polymer, Dyes Pigments 77 (2008) 415–426.
- 559 [41] M. Dalaran, S. Emik, G. Güçlü, T.B. İyim, S. Özgümüş, Study on a novel
560 polyampholyte nanocomposite superabsorbent hydrogels: Synthesis, characterization
561 and investigation of removal of indigo carmine from aqueous solution, Desalination 279
562 (2011) 170–182.
- 563 [42] T.W. Weber, R.K. Chakravorti, Pore and solid diffusion models for fixed-bed adsorbers.
564 AIChE J. 20 (1974) 228–238.
- 565 [43] D.G. Duff, S.M.C. Ross, D.H. Vaughan, Adsorption from solution: An experiment to
566 illustrate the Langmuir adsorption isotherm, J. Chem. Educ. 65 (1988) 815–816.
- 567 [44] C. Chipot, R. Jaffe, B. Maigret, D.A. Pearlman, P.A. Kollman, Benzene dimer: A good
568 model for π – π interactions in proteins? A comparison between the benzene and the
569 toluene dimers in the gas phase and in an aqueous solution, J. Am. Chem. Soc. 118
570 (1996) 11217–11224.
- 571 [45] C. Chang, L. Zhang, Cellulose-based hydrogels: Present status and application
572 prospects, Carbohydr. Polym. 84 (2011) 40–53.

- 573 [46] H. Kono, I. Oeda, T. Nakamura, The preparation, swelling characteristics, and albumin
574 adsorption and release behaviors of a novel chitosan-based polyampholyte hydrogel,
575 React. Funct. Polym. 73 (2013) 97–107.
- 576 [47] H. Kono, M. Zakimi, Preparation, water absorbency, and enzyme degradability of novel
577 chitin- and cellulose/chitin-based superabsorbent hydrogels, J. Appl. Polym. Sci. 128
578 (2013) 572–581.
- 579 [48] H. Kono, F. Otaka, M. Ozaki, Preparation and characterization of guar gum hydrogels
580 as carrier materials for controlled protein drug delivery, Carbohydr. Polym. 111 (2014)
581 830–840.
- 582 [49] M.I. Ugwoke, G. Kaufmann, N. Verbeke, R. Kinget, Intranasal bioavailability of
583 apomorphine from carboxymethylcellulose-based drug delivery systems, Inter. J.
584 Pharm. 202 (2000) 125–131.

1 **Figure captions**

2 **Fig. 1.** (A) Scheme illustrating the preparation of CAM from CMC; (B) structures of the anionic
3 dyes used in this study. The anionic carboxymethyl and the cationic quaternary
4 ammonium chloride groups of the CAMs can be located at any hydroxyl group of the
5 anhydroglucose unit of cellulose, but they are not shown in the figure.

6 **Fig. 2.** ^{13}C NMR spectra of CAMs in D_2O . The resonance assignments are also shown in
7 the figure. The C2s, C3s, C6s labels indicate C2, C3, and C6 centers in which
8 hydroxyl groups are substituted by anionic and/or cationic groups, respectively. The
9 subscripts (A) and (C) in the signal assignments denote anionic and cationic groups,
10 respectively. The baseline for the spectra is shown as dotted lines.

11 **Fig. 3.** (A) Effect of pH on the flocculation ability of AR13 by CAMs. The initial concentration
12 of the AR13 solution was 50 mg L^{-1} , and the amount of CAM added to the solution
13 was 2 mg. (B) Effect of temperature on the flocculation of AR13 by CAMs. The initial
14 concentration of AR13 solution was 50 mg L^{-1} , the amount of CAM added to the
15 solution was 2 mg, and the pH of the buffer medium was 7. (C) Effect of the
16 CAM4 dosage on the AR13 flocculation at pH 3. The initial AR13 concentration was 50
17 mg L^{-1} . All experiments were performed with 10 mL of dye solution in a 22 mL glass
18 vial.

19 **Fig. 4.** (A) Time dependence of flocculation of AR13 by CAMs 2–4 at pH 7. The initial
20 concentration of AR13 solution was 50 mg L^{-1} , and the volume of the dye solution
21 was 10 mL. The amount of each CAM added to the solution was 2 mg. (B) Pseudo-
22 first order kinetic plot and (C) pseudo-second order kinetic plot for the flocculation of
23 AR13 by the CAMs.

24 **Fig. 5.** (A) Effect of initial dye concentration (C_0) on the flocculation ability at equilibrium
25 (q_e) of CAM4 for (A) AR9, (B) AR13, and (C) AB92 at pH 3 and 7. All experiments
26 were performed in 10 mL of each dye solution, and the amount of CAM added to the
27 dye solution was 1 mg.

28 **Fig. 6.** (A) Langmuir isotherm and (B) Freundlich isotherm for the flocculation of AR9, AR13,
29 and AB92 at pH 3 and 7 using CAM4 as a flocculant.

30 **Fig. 7.** (A) Photographs of AR9, AR13, and AB92 solutions before and after flocculation by
31 CAM4 at pH 3, and (B) SEM image of the flocs formed from these anionic dyes.

1 **Tables**

2

3 **Table 1** DS_C, DS_A, and yields of CAm_s1–4.

CAm	DS _C	DS _A	Yield (%) ^a
1	0.26	0.60	1.02 (89)
2	0.66	0.60	1.28 (90)
3	0.83	0.60	1.36 (88)
4	1.08	0.60	1.58 (92)

4 ^aYield (%) was determined by Eq. (5).

5

6

7

8 **Table 2** Kinetic parameters (Eqs. 7-8) for flocculation of AR13 by CAm_s2–4 at pH 7 and 298 K.

CAm	$q_{e,exp}$ /mg g ⁻¹	Pseudo-first order model			Pseudo-second order model		
		k_1 /min ⁻¹	$q_{e,cal}$ /mg g ⁻¹	R ²	k_2 /g mg ⁻¹ min ⁻¹	$q_{e,cal}$ /mg g ⁻¹	R ²
2	16.0	0.069	8.42	0.928	0.059	16.7	0.999
3	41.2	0.056	18.0	0.803	0.023	43.7	0.998
4	92.0	0.066	35.2	0.811	0.010	95.8	0.999

Table 3. Langmuir and Freundlich adsorption isotherm coefficients (Eqs. 9-11) for the flocculation of cationic dyes by CAm 4 at pH3 and 7.

Dye	pH	Langmuir adsorption isotherm				Freundlich adsorption isotherm		
		$C_0 / \text{mg g}^{-1}$	$b / \text{mg}^{-1} \text{L}$	R_L	R^2	$K_F / \text{mg L}^{-1}$	n^{-1}	R^2
AR9	3	558	1.13×10^{-2}	0.128	0.996	12.2	0.719	0.963
	7	270	5.10×10^{-3}	0.246	0.998	1.57	0.908	0.966
AR13	3	450	1.29×10^{-2}	0.115	0.997	12.8	0.655	0.942
	7	195	6.64×10^{-3}	0.201	0.997	3.24	0.711	0.977
AB92	3	457	6.33×10^{-3}	0.208	0.998	5.12	0.820	0.957
	7	140	4.62×10^{-3}	0.255	0.998	1.26	0.791	0.991

Figure 1

(A)

(B)

Figure 2

Figure 3

(A)

(B)

(C)

Figure 4

Figure 5

(A)

(B)

(C)

Figure 6

Figure 7

(A)

AR9

AR13

AB92

(B)

AR9

AR13

AB92