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Understanding the effect of carbon dots with size variation on collagen

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The unique optical properties of carbon dots (CDs) offer a wide variety of applications both in chemical and biological environments. The interaction of CDs with biological molecules such as proteins and DNA has been studied extensively. In the present study, the interaction of two different sizes of CDs with collagen, a fibrillar protein, has been investigated. The different sizes of CDs have been characterized using various techniques such as HR-TEM, SEM, and zeta potential. Upon interaction with CDs, the triple helical nature was not altered and R_{pn} value was similar to the control collagen. The influence of size on the fibrillation of collagen process is significantly impacted with photoirradiation process. Similarly, in DSC experiments, the CDs destabilized the collagen with ΔTm value of 16±1°C but with photoirradiation, the stability of the collagen is improved but less than the control collagen. In EPR studies upon photoirradiation, a significant amount of generation of hydroxyl radicals is observed. The gel electrophoresis studies show that the presence of CDs did not inhibit the collagenase activity without or with photoirradiation. The data from the present study clearly reveals that the carbon dots can be utilized with varied drug loading on collagen that can be utilized for bioengineering applications.

Keywords: Carbon dots, Fibrillation, Photoirradiation, Radicals

Type I collagen is the most common protein found in the body and serves as a building block in tissues[1]. The assembly of the collagen fibrils dictates the biological responses as well as the overall mechanical properties of the biomolecule[2]. Numerous studies have been reported on improving the stability/mechanical strength of the collagen offered by either crosslinking/interacting with various small molecules that range from nanoparticle to metal complex to polyphenolic systems[3, 4]. Further, biocomposites made of collagen loaded with drugs or different metal systems have been explored for tissue engineering[5-7].

One of the emerging nanoparticles, *i.e.* carbon dots have been studied for its various chemical and biological applications. Due to the excellent photoluminescent behaviour of carbon dots[8], it has been extensively used as sensors for various molecules[9-11]. In addition, these carbon dots in the form of carbon quantum dots have been used in photoreduction of heavy metal ions, optoelectronic devices, bioimaging, *etc.*[12-14]. Studies on carbon dots based bioscaffolds have shown promising outcome in bone tissue engineering. Gogoi et al. studied the CDs-peptide composites implanted in polyphenol and polyurethane matrix for *in vivo* bone regeneration [15,16]. Due to the low toxicity and ease of preparation of CDs[17-19], CDs play significant role in tissue engineering. Lu et al., studied the injectable hydrogel containing CDs-genipin for the application in cartilage regeneration[20].

The interaction of CDs with DNA induces conformational change from right handed to left handed helix[21], while carbon nanotubes leads to B-A conformation[22]. The binding of these CDs with positive charge to GC rich region induces the transition. Interestingly, the work on CDs with varied size or photoluminescence property exhibiting any influence of the collagen structure has not yet been explored. In this study, the interaction of two CDs (Red and Yellow) with collagen was studied. Based on the sizes, these two carbon dots altered the fibrillation process under the photoirradiation.

Experimental Section

o-Phenylenediamine and potassium chloride were purchased commercially and used as such. Type I

collagen was extracted from rat tail tendon in 0.5 M acetic acid solution described in established procedure[23]. All other solvents used in this study was purchased and used as received.

Preparation of red and yellow carbon dots

For the synthesis of the red and yellow carbon dots, the precursors, *o*-phenylene diamine (25mmol) and KCl (5mmol) were mixed[24]. The mixture was taken in mortar and was grounded together for 15 minutes. The ground mixture was then added to a Teflon-lined stainless-steel autoclave and heated for 10 hours at 200°C to obtain red emissive carbon dots and heated for 8 hours at 200°C to obtain yellow emissive carbon dots. The obtained carbon dots were washed with ethanol to double distilled water ratio of 1:10 thrice to remove the impurities. The remnants were centrifuged in ethanol at 10,000 rpm. The supernatant was used for further characterization.

Characterization of carbon dots

Transmission electron microscopic (TEM) analysis for the carbon dots was carried out in JEOL Japan, JEM-2100 Plus HRTEM. The carbon dots dispersed in ethanol were coated in the copper grid and dried for the TEM characterization. Zeta analysis was carried out to find the charges on the carbon dots using Anton PaarLitesizer 500.The UV spectra of the carbon dots were obtained from JASCO-V-750 spectrophotometer. The emission spectra of the carbon dots were recorded in Hitachi F-7000 spectrofluorimeter equipped with Peltier temperature controller. Powder XRD was carried out for the carbon dots in Rigaku Miniflex II Desktop X-ray diffractometer.

Interaction of carbon dots with collagen

Fibrillation kinetics assay

The effect of red and yellow fluorescent carbon dots on collagen fibril formation was measured at 313 nm using JASCO-V-750 spectrophotometer equipped with Peltier temperature controller. Collagen and carbon dots (in the weight ratio of 1:0.2 and 1:1, respectively) were incubated for 24 h. The fibril formation of collagen was determined by bringing the final concentration to 0.3 mg/mL of collagen using 50 mM phosphate buffer and 75 mM sodium chloride at 25°C. The turbidity was measured after adjusting *p*H to alkaline by using 0.5 M NaOH. The turbidity ($t_{1/2}$) was used to determine the rate of fibril formation. Control experiments were performed on collagen either exposed with or without photoirradiation at 290 nm for 30 min.

Differential scanning calorimetry studies (DSC)

For DSC studies, rat tail tendon was used. The analysis was carried out in NETZSCH DSC-214 at a 2°C/min heating rate under a nitrogen atmosphere. Collagen tendons were treated with red and yellow emissive carbon dots at a different ratio mentioned in the fibrillation assay and incubated for 24 h. Control experiments were carried out for the photoirradiated and non-photoirradiated collagen.

Circular dichroic (CD) studies

The influence of red fluorescent carbon dots and yellow fluorescent carbon dots on the secondary structure of collagen was studied using JASCO J-815 spectropolarimeter under a nitrogen atmosphere. Collagen in 10 mM acetate buffer was incubated with the carbon dots in the weight ratio as used in the fibrillation assay, and the measurement was carried out using a quartz cell path length 0.1 cm at 25°C.

FTIR analysis

Fourier-transform infrared spectroscopy of collagen fibril in the presence and absence of carbon dots was analyzed using BRUKER ALPHA II. For this analysis, the collagen fibrils were prepared similar to the fibrillation process mentioned above. The spectra were recorded for the lyophilized fibrils over the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}.

EPR analysis

EPR analysis of the photoirradiated and non-photoirradiated carbon dots was carried out in JEOL Resonance JES-X310. DMPO was used as the spin-trapping agent for the formed radicals. The control experiments were carried out for collagen and photoirradiated collagen. The ratio used for this analysis was 1:0.2 of collagen and carbon dots, respectively.

Results and Discussion

Preparation and characterization of carbon dots

The preparation of red and yellow carbon dots was carried out by following the reported literature[24]. The prepared CDs were characterized using various spectroscopic techniques. The carbon dots exhibited emission wavelength of 704 nm upon excitation at

210 nm and labelled the CDs as RCDs while the one with shorter reaction time treated CDs exhibit a yellow emission at 556 nm when excited at 207nm and labelled as YCDs (Fig. S1). TEM analysis of CDs showed that RCDs have an average size of 8-10 nm while YCDs with less than 2 nm of size (Fig. S2). The zeta potential of the RCDs and YCDs were shown to be –4 and 0 mV, respectively (Fig. S3). Powder XRD data shows that both prepared CDs exhibit 002 plane at 20° (Fig. S4).

Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) data on the prepared CDs reveals the presence of increased amount of free radical species (·OH)[25] after interaction with DMPO for the photoirradiated sample while in the normal experimental condition the presence of radical is limited as shown in Fig. 1.

Turbidity measurements

The impact of two different sizes of carbon dots on collagen was studied to understand to what extent the carbon dots affect the fibrillation and stability of the collagen. Fibrillogenesis involves a two-step process, *i.e.* (i) the aggregation of individual helical molecules into nuclei and (ii) the growth of nuclei into fibrils. The efficiency of crosslinking and fibrillation rate is generally monitored by a change in turbidity (due to growth of fibril formation) at 313 nm. The effect of a small molecule on the fibril formation was determined by its final turbidity (Δh) and the time taken to reach half the value of its turbidity ($t_{1/2}$)[26, 27]. The interaction of CDs with collagen was carried out for both with and without photoirradiation. As seen from Fig. 2 and Table 1, collagen control and CDs treated collagen

Table 1 — Fibrillation kinetics data with $t_{1/2}$ values and rpn values of collagen in the absence and presence of carbon dots with and without photoirradiation

Samples	$t_{1/2}$ (min)[a]	rpn
Control	1.5	0.12
+RCDs	1.4	0.12
+hv	7.7	0.17
+YCDs	1.4	0.13
+hv	3.6	0.12

[a]Final turbidity (Δh) is 0.5 for all systems; Experiments were carried out in triplicate and the values are within the error of 5%.

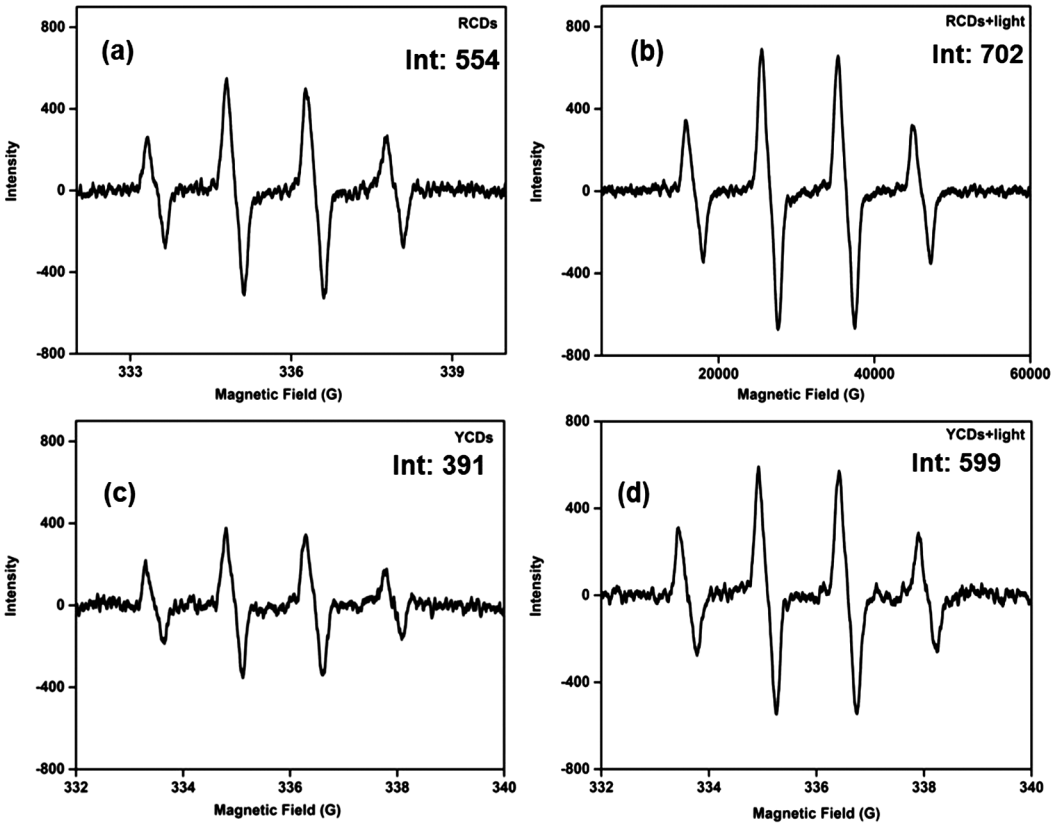

Fig. 1 — EPR spectra of red and yellow carbon dots: a) red carbon dots, b) photoirradiated red carbon dots, c) yellow carbon dots, and d) photoirradiated yellow carbon dots.

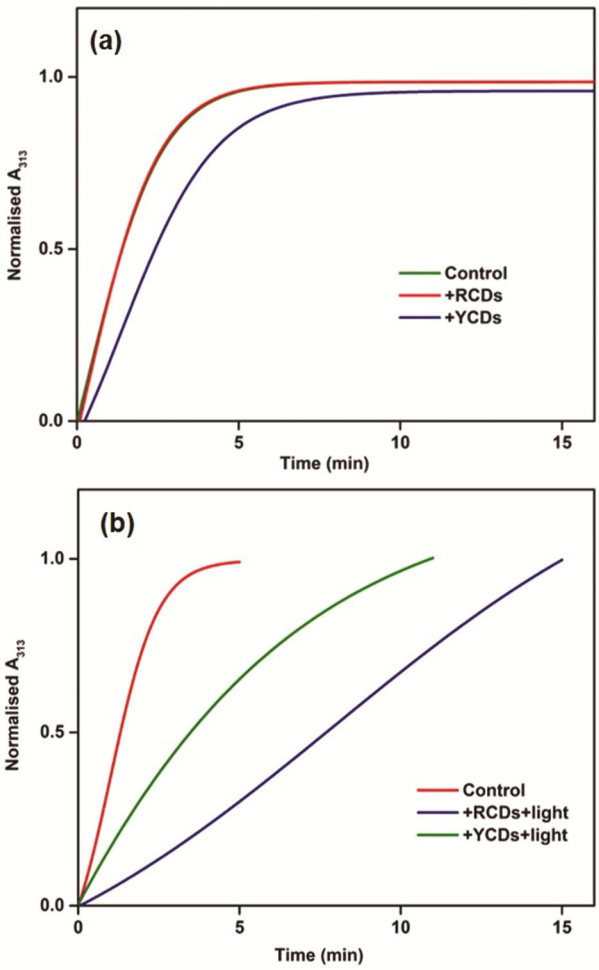

Fig. 2 — Fibrillation kinetics of control collagen and carbon dots treated collagen with and without photoirradiation (a) Red and yellow carbon dots treated control collagen (b) Red and Yellow carbon dots treated control collagen with photoirradiation.

has the similar $t_{1/2}$ of around 1.5 min. However, upon photoirradiation, the fibrillation process was slightly faster than non-irradiated sample but in the case of RCDs, the fibrillation process was totally inhibited while with YCDs, the fibrillation was delayed by three-fold. Generally, small molecules which crosslinks the collagen inhibits or delays the fibrillation process[26-32]. In certain cases, there are molecules such as ruthenium conjugated gold nanoparticles upon photoirradiation conditions enhances the fibrillation process compared to the control collagen like high pH, temperature, enzymes *etc.* [29,33]. In the present scenario, the carbon dots alone did not impact the fibrillation process but with photoirradiation, due to the generation of free radicals might have facilitated the crosslinking and delayed the fibrillation process and is supported by EPR data,

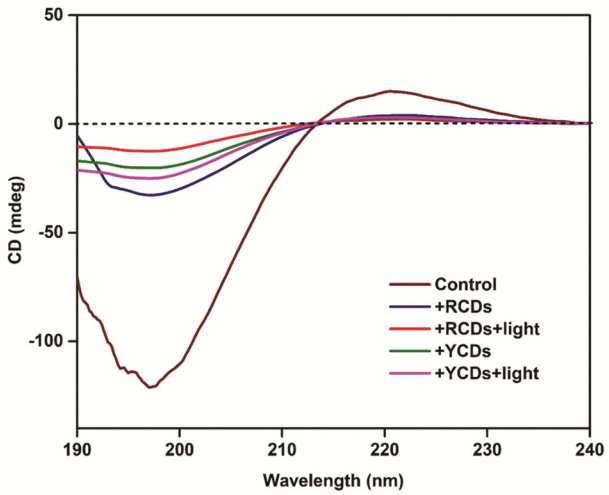

Fig. 3 — CD spectra of control collagen and carbon dots treated collagen (with and without photoirradiation).

in which the production of free radical increases with photoirradiation process.

Circular dichroic studies

Circular dichroic spectra were recorded in the far UV region of 280 nm –190 nm to determine the effect of carbon dots on the secondary structure of collagen.To determine any triple helical structural perturbation that occur with treatment of collagen with CDs under dark or photoirradiated condition, circular dichroic spectra was recorded. Interestingly, when compared to control collagen, there was no perturbation observed for all the samples and ratio of positive to negative peak (R_{pn}) was similar to the control as given in Fig. 3 and tabulated in Table 1. From the spectra, the positive peak is at 221 nm and a negative peak at 197 nm with a crossover point at 214 nm[34].

Thermal stability studies

Further, calorimetric studies were performed for both non-radiated and irradiated collagen with CDs to determine the stability. The transition temperature of collagen was observed at 60°C while upon treatment with RCD and YCD, the collagen structure was destabilized by 20 and 17° C, respectively. Upon photoirradiation, the control collagen itself exhibited better stability of 15°C to 75°C while with RCD and YCD, the temperature increased by 11 and 16 °C, respectively. The increase in stability of collagen upon photoirradiation clearly shows that the possibility of crosslinking by tyrosine radicals (Fig. 4.). With the RCD and YCD, a similar increase in transition

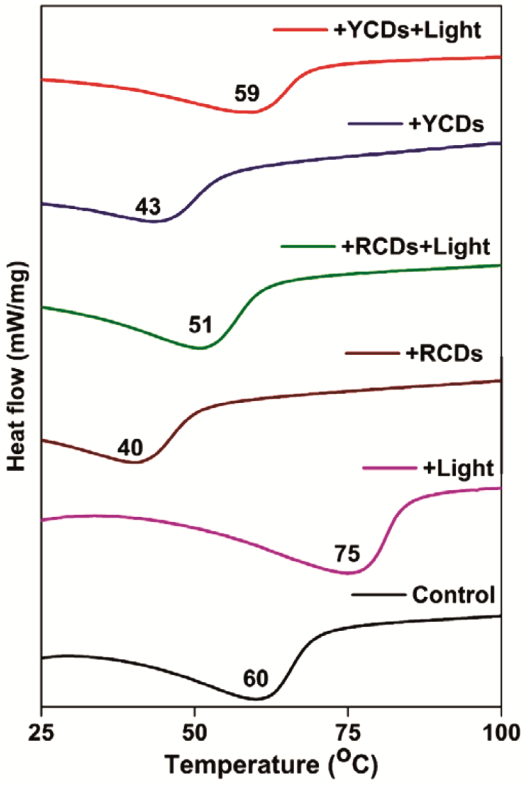

Fig. 4 — DSC analysis for control collagen and carbon dots treated collagen with and without photoirradiation

Table 2 — FT-IR values for control collagen and carbon dots treated collagen (with and without photoirradiation)

Samples	Wavenumber (cm^{-1})		
	ν_{N-H}	Amide I	Amide II
Control	3359	1638	1550
+RCDs	3312	1632	1555
+hv	3359	1638	1553
+YCDs	3361	1643	1555
+hv	3345	1633	1553
Control+light	3345	1637	1559
RCDs	-	-	-
YCDs	-	-	-

temperature occurred and could be due to synergistic effect of ROS created by CDs and collagen.

FT-IR studies

The data from FT-IR studies also reveal that the addition of carbon dots affects the backbone of the collagen in which the stretching frequency of N-H shifted from 3359 in control collagen to 3312 when it is interacted with RCDs while YCDs did not alter (Table 2). Upon photoirradiation, untreated collagen itself showed a shift of 14 cm^{-1} while with RCDs shift of 47 cm^{-1} from 3312 to 3359 cm^{-1} was observed.

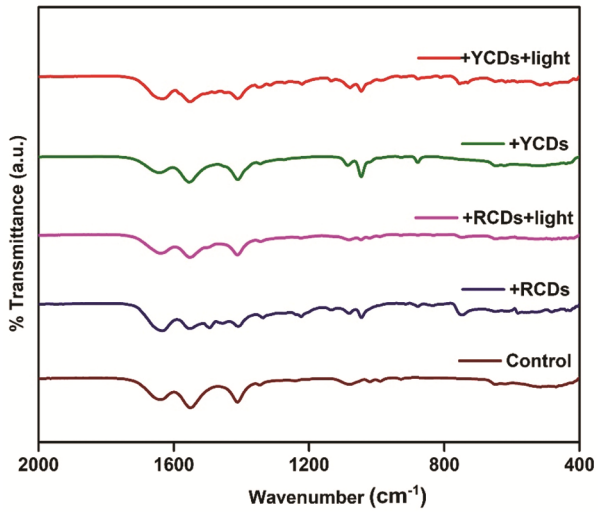

Fig. 5 — FT-IR analysis for control collagen and carbon dots treated collagen with and without photoirradiation.

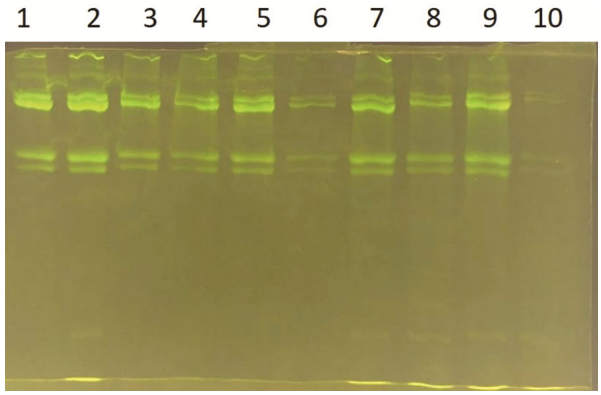

Fig. 6 — SDS-PAGE gel from left to right: (Lane 1) Control, (2) control + collagenase, (3) RCDs + control, (4) RCDs + control + light, (5) YCDs + control. (6) YCDs + control + light, (7) RCDs + control + collagenase, (8) RCDs + control + collagenase + light, (9) YCDs+ control + collagenase, (10)YCDs + collagen + collagenase + light.

With respect to amide I and II, there is no significant shift in the stretching frequency (Fig. 5.). The results from the FT-IR study further emphasizes on the size of the carbon dots that interacts with collagen particularly RCD compared to YCDs has greater implications as evident from fibrillation assay as well.

Gel studies

To understand the role of the size of CDs on the collagenase activity, collagen treated CDs with or without photoirradiation was treated with collagenase and its cleavage pattern was studied. Upon treatment with collagenase, collagen gets fragmented to smaller fragments (Fig. 6). The irradiation of CDs did not bring any cleavage on collagen. Further, the treatment of

collagenase on the photoirradiated collagen with or without CDs did not vary from the control collagen cleavage pattern. In contrast, photoirradiated ruthenium complex conjugated gold nanoparticles treated collagen did not undergo enzymatic digestion after addition of collagenase[29]. Depending on the nature of Ru(II) complex conjugated with gold nanoparticle, the fibrillation assay as well as the collagenolytic activity differs. The data from the gel electrophoresis clearly reveals that carbon dots have minimal effect on the structural stability of collagen and thus leads to the collagen cleavage upon enzymatic digestion.

Conclusions

Taken together, the results from this study clearly reveals that carbon dots with different size exhibit (i) varying degree of inhibition on collagen fibrillation; (ii) destabilizes the collagen by 15-17°C compared to control collagen, while on irradiation the stability of collagen increases; (iii) clear shift in N-H backbone from FT-IR analysis (iv) no perturbation occurs to the collagen triple helical structure (v) CDs have negligible impact on collagenase activity. The overall results from the work indicated that the size of the carbon dots has significant implications on the collagen structure and also proves to be an interesting system for tissue engineering studies to understand how the different size of carbon dots makes drug efficient and the cause is yet to ascertain.

Supplementary Information

Supplementary information is available in the website http://nopr.niscpr.res.in/handle/123456789/58776.

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References

1 Maynes R, Structure and Function of Collagen Types, (Elsevier) 2012.
2 Delgado L M, Fuller K & Zeugolis D I, *Tissue Engineering Part A*, 23 (2017) 1064.
3 Kandamachira A, Selvam S, Marimuthu N, Kalarical S J & Nishter N F, *Soft Materials*, 13 (2015) 59.
4 Wang D, Ye J, Hudson S D, Scott K C, Lin-Gibson S, *J Coll. Int. Sci*, 417 (2014) 244-249.
5 Kanungo I, Fathima N N, J R Rao, B U Nair, Materials Science and Engineering: C, 33 (2013) 4651-4659.
6 Anghel N, Dinu V M, Verestiuc L, Spiridon I A, *Polymers*, 13 (2021) 1845.
7 M G Albu, I Titorencu, M V Ghica, chapter 2011. Collagen-based drug delivery systems for tissue engineering.DOI: 10.5772/22981
8 Zhu S, Song Y, Zhao X, Shao J, Zhang J, Yang B, *Nano Res*, 8 (2015) 355-381.
9 Zhu S, Meng Q, Wang L, Zhang J, Song Y, Jin H, Zhang K, Sun H, Wang H & Yang B, *Angew Chem Int Ed*, 52 (2013) 3953.
10 Sun X & Lei Y, *Trends Analytical Chem*, 89 (2017) 163.
11 X Lin, M Xiong, J Zhang, C He, X Ma, H Zhang, Y Kuang, M Yang, Q Huang, *Microchemical J*, 160 (2021) 105604.
12 Y Li, Z Liu, Y Wu, J Chen, J Zhao, F Jin & P Na, *Applied Catalysis B: Env*, 224 (2018) 508.
13 H Li, X Yan, D Kong, R Jin, C Sun, D Du, Y Lin, G Lu, Nanoscale Horizons, 5 (2020) 218-234
14 T Feng, S Tao, D Yue, Q Zeng, W Chen, B Yang, *Small*, 16 (2020) 2001295.
15 J Han, J Hong, H Lee, S Choi, K Shin, M Gu, S -H Kim, *Eur Poly J*, (2023) 112354
16 S Gogoi, S Maji, D Mishra, K S P Devi, T K Maiti, N Karak, *Macromolecular Biosci*, 17 (2017) 1600271.
17 X Yao, R E Lewis, C L Haynes, *Acc Chem Res*, 55 (2022) 3312-3321.
18 M Havrdova, K Hola, J Skopalik, K Tomankova, M Petr, K Cepe, K Polakova, J Tucek, A B Bourlinos, R Zboril, *Carbon*, 99 (2016) 238-248.
19 C Xia, S Zhu, T Feng, M Yang, B Yang, *Advanced Science*, 6 (2019) 1901316.
20 Z Lu, S Liu, Y Le, Z Qin, M He, F Xu, Y Zhu, J Zhao, C Mao, L Zheng, *Biomaterials*, 218 (2019) 119190
21 L Feng, A Zhao, J Ren, X Qu, *Nucleic Acids Res*, 41 (2013) 7987.
22 X Li, Y Peng, X Qu, *Nucleic Acids Res*, 34 (2006) 3670-3676.
23 N Thangavel, I Jayakumar, M Ravichandran, V V Ganesan, B U Nair, *Spectrochim. Acta Part A: Mol.Biomol. Spec.*, 215 (2019) 196.
24 H Ding, X -X Zhou, Z -H Zhang, Y -P Zhao, J -S Wei, H -M Xiong, *Nano Res*, (2022) 1-8.
25 S Sundarapandiyan, T S Renitha, J Sridevi, P Saravanan, B Chandrasekaran, G B Raju, *Chem Eng J*, 313 (2017) 1112-1121.
26 H Notbohm, S Mosler, P K Müller, J Brinckmann, *Int J Bio Macromol.*, 15 (1993) 299.
27 B R Williams, R A Gelman, D C Poppke, K A Piez, *J Biol. Chem*, 253 (1978) 6578.
28 M Visalatchi, B Madhan, G Shanmugam, N-Vanillyl nonanamide, *Int J Bio Macromol.*, 156 (2020) 1146
29 N Thangavel, I Jayakumar, M Ravichandran, V Vaidyanathan Ganesan, B U Nair, Photocross linking of collagen using Ru(II)-polypyridyl complex functionalized gold nanoparticles, *Spectrochim. Acta Part A: Mol. Biomol Spec*, 215 (2019) 196-202.
30 P Anithabanu, V G Vaidyanathan, *Int J Biol Macromol*,190 (2021) 56-60.
31 P David Dayanidhi, P Anithabanu & V G Vaidyanathan, *Biophys. Chem*, 292 (2023) 106917.
32 Jaikumar D, Baskaran B & Vaidyanathan V G, *Int J Biol Macromol*, 96 (2017) 429.
33 Kezwon A & Wojciechowski K, *Colloids Surf. A: Physicochem. Eng. Asp.*, 460 (2014) 168.
34 Piez K A, Sherman M R, *Biochemistry*, 9 (1970) 4129.