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Visible auto-fluorescence in biological fluids as biomarker of pathological processes and new monitoring tool

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Comparative optical study of biofluids (serum, urine, hemodialysate) by concentration change of endogenous visible fluorescence substance (VFS) has been carried out. Biofluids were collected from chronic kidney diseases (CKD) patients (Pts) as well as from healthy controls (HCs). Excitation/emission spectra are similar for all samples of biofluids differing only in intensity, which is higher for CKD Pts. Strong similarity enables the study of given biofluids from a united physical platform, proposed earlier, i.e., as nanoparticles approach. Specific spectral redistribution of visible fluorescence (VF) intensity as a result of dilution is revealed. The concentration change of VFS by dilution of samples manifests in nonlinear dependences in the scales "VF intensity–concentration" for serum and urine and in perfect linearity for hemodialysate (HD). The latter fact can be used in monitoring of hemodialysis procedure.

Keywords: Serum; urine; hemodialysate; nanoassociates; graphene.

1. Background

Biological fluids (serum, urine, hemodialysate (HD)) exhibit auto-fluorescence spreading over the whole visible region.[1–6] The main parameters were given in Refs. 2 and 3 as $\lambda_{excmax}/\lambda_{emmax} \approx 320/420$ nm but they need specific rectification because of dependence of emission on the excitation energy ("red shift (RS) phenomenon").[1,5] This visible fluorescence (VF) is not directly connected with UV fluorescent proteins and can be observed in protein-free fractions of biofluids.[6] Moreover, the VF substance (VFS) is ninhydrin-negative.[3] The intensity of VF in serum is a reliable indicator of chronic kidneys diseases (CKD) being higher in Pts compared with healthy controls (HCs).[1–4,7,8] There are also other manifestations of possible pathological origin of VF as it can be concluded from experiments with artificial brain necrosis in rats.[9] We have found a correlation between the normalized VF intensity in HDs

and concentration of C-reactive protein (CRP) in CKD Pts' blood.[10]

Many efforts have been undertaken to identify the VFS but to our best knowledge without clinically conclusive result.[1–4,7,8,11] Since VFS is present not only in CKD Pts' bodies but in those of HC too,[1–7] the knowledge of its nature and the role it plays in body seems to be a challenge not only for CKD Pts care but for metabolomics as a whole. Earlier we have successfully applied as an investigation method carbon nanoparticles (CNP) approach and found a plenty (>10) of strong similarities in optical absorption, fluorescence and reactivity properties of VFS in biofluids and CNP aqueous solutions.[5,6,12,13] Nevertheless, the constituent molecules and driving mechanism of their self-assembly into rather typical entities in biofluids still require further efforts to be clarified.

Concrete need exists also for clear-cut information on concentration, quenching and self-quenching effects in auto-fluorescence of biofluids as multi-fluorophore systems. In relation to urine it was claimed earlier that there exists a principal concentration problem, namely — "fully different fluorescence spectra of the same material caused only by the dilution of the sample".[14] The same can hold for blood serum or plasma. Intentional change in the concentration of chromophores or fluorophores is a classical method to study association (self-assembly) processes in solutions starting with the pioneering Scheibe's work.[15] Nevertheless, the systematic concentration-change experiments in serum, urine and HD in relation to VF have not been done.

Since the VF intensity seems to be the main indicator in diagnostics or monitoring (other VF parameters are not much specific or selective), it is evident that the VF concentration dependences must be taken into account accurately. At the same time such data are very scarce[14] or were not obtained at all.[2,3,7,8] Common fluorescence approach to three different biofluids (serum, urine, HD) proposed in Refs. 2 and 3 is a rather heuristic one but in those and following papers[4,7,8,11] the correct excitation/emission spectra of biofluids (mainly sera) were not presented. Therefore, it is impossible on this base of data[2,3] to decide how strong spectroscopic similarities/differences for the biofluids (maybe originating from one and the same body) can be observed and used as parameters for further analysis and practical purposes.

2. Aim

The aim of this work was to continue the systematic comparative study of VF and VFS in different CKDs' and HCs' biofluids (urine, serum, HD).[5] Dilution of urine and serum samples, i.e., the change of VFS concentration in the widest possible range was used as the main tool in experiments which were accompanied by measurements of absorption, fluorescence and fluorescence excitation spectra in the region of 200–700 nm. Secondly, it was aimed to propose and realise (at least in a preliminary version) some practical use of VF in diagnostics or monitoring of hemodialysis.

3. Subjects and Methods

Samples of urine, serum and HD were collected in the hemodialysis department of Tartu University Hospital (TUH) from G5 CKD Pts: 17 males, 13 females, age range 30–81, age in average — 63. Serum and urine were collected also from healthy persons (12) who formed the control group (HCs). Measurements and other treatments were performed mostly not later than 2 h after sampling. In other cases, samples were stored at -20°C. Some samples of urine were centrifuged before fluorescence measurements, but we have observed no remarkable differences in the spectra depending on the centrifugal procedure. Biochemical analysis were carried out in the United Laboratory at the TUH.

Part of measurements was performed with proteins-free samples. This was achieved using fractionation of sera in PD-10 desalting columns with Sephadex^{TM} G-25 Medium (GE Healthcare) with the cut-off $M < 5$ kDa. The VFS was eluted mostly in the middle mass molecules fraction called "1" next to the proteins fraction "0". This procedure allowed us to get rid of very intense UV fluorescence ($\lambda_{emmax} \approx 355$ nm) of proteins in sera which can disturb the measurement of weaker VF. Such a purification shows clearly that BFS is not tightly bound to large proteins.

Distilled water or blank dialysates SW 139 A (B Braun) were used for diluting biofluids. Blank dialysate is an analogue of physiological fluids and is being used as a working liquid in hemodialysis. No meaningful difference was observed by changing the dialysate diluter against distilled water and therefore the most part of experiments was done with

distilled water. Maximal range of dilution was 100–0.01%.

Most of fluorescence measurements were performed at room temperature with the computer-steered 3D-fluorimeter NarTest NTX 2000 (LDI Tallinn) operating in the front geometry. This geometry allows to diminish down to the negligible values the inner filter effects in the emission spectra measurements. Scanning steps were usually 5 nm for excitation as well as for emission registration. All spectral corrections are being done automatically in this apparatus besides the absorption level correction of exciting light. The optical absorption of biofluids, especially in diluted samples, may be rather low in the near UV ($\lambda > 350$ nm) and visible region ($k < 1\,\mathrm{cm}^{-1}$), therefore the loss of exciting light must be taken into account. The absorption spectra were measured with the spectrophotometer Jasco V-550 and afterward the absorption correction of excitation spectra was done. The corrections were very essential for excitation spectra of all HDs and strongly diluted urine and serum.

4. Ethics

The study has been approved by the Ethics Committee on Human Research of the Tartu University, Estonia (protocol no 219/M-19; 2012).

5. Results

We have measured the series of VF spectra of serum and urine usually by their gradual dilution with appropriate steps (factors 2 or smaller). For the control purposes we have moved also in the opposite direction increasing the concentration in already diluted samples by adding the same whole fluids (urine). No remarkable difference in dependencies on the direction of concentration change was observed.

5.1. *Fluorescence and excitation spectra*

In Fig. 1, we demonstrate the emission spectra of a CKD Pt's serum, urine and HD at the excitation of 360 nm and in Fig. 2 absorption corrected excitation spectra for emission of 430 nm, accordingly. The main emission maxima are located at 435 nm. The normalized emission spectra of urine and HD coincide fairly with each other what is a natural result, since HD is essentially a diluted urine. This observation does not support the hypothesis[14] that dilution can change the emission spectra of urine always very drastically and unpredictably. In our opinion, more typical and important is the reproducibility of the main spectral features when excitation energies are taken correctly into account. Many measurements with different samples have demonstrated that VF intensity in serum is always higher for CKD Pts than for HCs but this difference is not so large as it was stated in Refs. 3 — 80 times in average. The extreme difference we have observed is approximately 10. Probably, this reflects the great progress in hemodialysis achieved during last 30–40 years. For urine, from Pts with remained renal function, the difference also seems to exist but not so large and it is masked

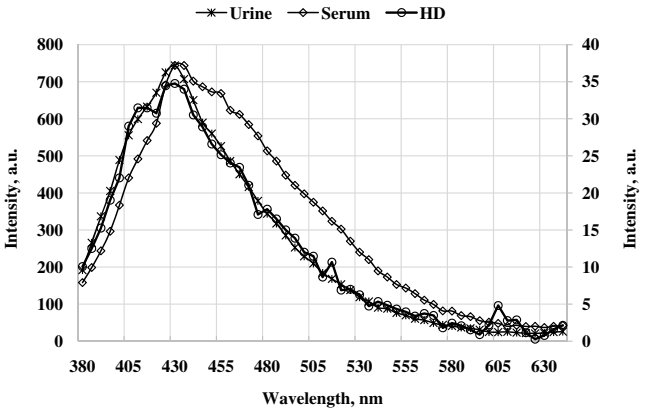

Fig. 1. A CKD Pt's biofluids fluorescence spectra measured at $\lambda_{exc} = 360$ nm. Serum and urine spectra are normalized at 435 nm. For HD spectrum the right-hand axis is valid.

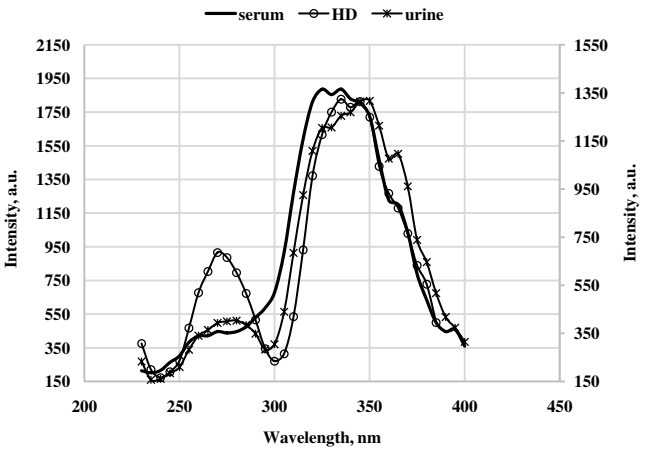

Fig. 2. Excitation spectra for $\lambda_{em} = 430$ nm in a CKD Pt's whole serum (the left intensity axis) and normalized spectra for whole urine (HC) and HD.

by quenching effects in undiluted samples (see Sec. 5.3).

The excitation band is wide and complex with the center of gravity at 335–340 nm and weak maxima at 325, 335, 350 and 365 nm. The excitation band at ≈ 280 nm belongs to the longwave tail of emission (typically $\lambda_{emmax} \approx 355$ nm) of residual proteins and peptides and will not be considered here in detail. Earlier, it was referred in Refs. 2 and 3 to $\lambda_{excmax}/\lambda_{emmax} \approx 320/420$ nm for all biofluids as the representative parameters. It must be taken into account, however, not only the complexity of emission/excitation bands but, first of all, the RS effect.[1,5] RS or the shift of emission maximum into the long wavelength direction by lowering of excitation energy is especially pronounced at $\lambda_{exc} > 350$ nm and makes the unambiguous determination of $\lambda_{excmax}/\lambda_{emmax}$ impossible in principle (see serum fluorescence spectra in Ref. 1 and the set of excitation spectra in Fig. 3 for different combination of $\lambda_{exc}/\lambda_{em}$ for urine in Ref. 4). At the same time, the RS of VF with close characteristic parameters[5] is one more phenomenon which unites the serum, urine and HD together.

The emission spectrum of serum is somewhat expanded and is more intensive in the long wavelength region in comparison to urine and HD spectra (Fig. 1). According to our nanoparticles approach,[6,12,13] this can be explained by the size and mass filtration occurring in the kidneys or dialysis filter accordingly. The filtration precludes the larger fluorescent moieties which have more reddish emission than the smaller ones[6] to penetrate the membranes and get into urine or HD. We have observed that fractionation of serum in the PD-10 columns resulted in the narrowing of emission spectra and in its shift into the blue direction. For urine and HD, this effect was not seen.

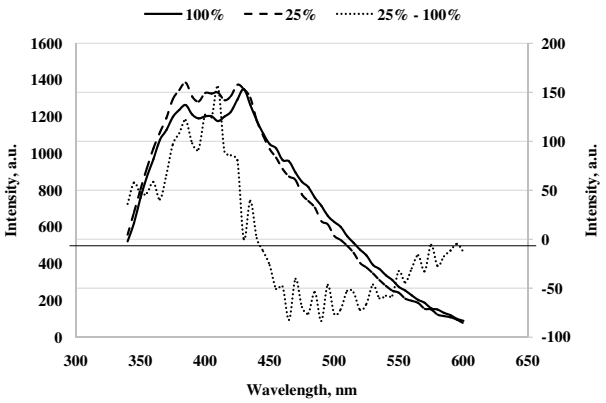

Fig. 3. Fluorescence spectra and their subtraction for the whole 100% and diluted to the level 25% serum from a CKD Pt. Excitation 320 nm. For the difference spectrum → the right-hand axis.

5.2. *Mirror reversed spectral changes by dilution*

At the higher energy excitations $\lambda_{exc} = 320 \pm 20$ nm the local emission maxima at 385 ± 5 nm and 410 ± 5 nm for all biofluids in this study have appeared. In Fig. 3, the emission spectra at $\lambda_{exc} = 320$ nm of the whole and diluted (25%) serum are drawn and normalized at $\lambda = 430$ nm. The picture shows clearly that in the long wavelength tail, the emission is more intensive for the whole sample than for diluted one and in the shorter wavelength region ($\lambda < 430$ nm) the difference is vice versa. The third curve (dashed line) shows the spectral difference of these two spectra and it becomes evident that by dilution, we diminish the fluorescence with $\lambda > 430$ nm and enhance the emission intensities at $\lambda < 430$ nm.

In Fig. 4, the light sums are depicted for "reduced" long wavelength emission (430–600 nm) and for "increased" blue emission (340–430 nm) versus the dilution level. The light sums and exchange dynamics "reduced" ↔ "increased" emission quantitatively grow by higher dilution levels. The appearance that "increased" light sums are smaller than "reduced" ones is probably an artifact since the emission measurements were limited at $\lambda = 340$ nm when $\lambda_{exc} = 320$ nm.

This mirror-reversed effect can be observed at the other λ_{exc} in the excitation range with the peculiarity that the "reduced" emission maximum

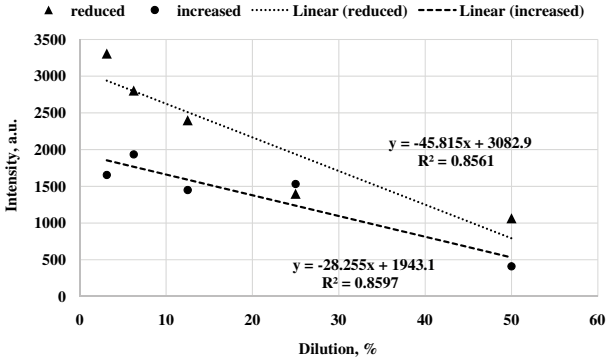

Fig. 4. Reduced (triangles) and increased (circles) light sums as the result of a CKD Pt's serum dilution to the levels of residual VFS given in percentage.

shifts to the smaller energies with the lowering of excitation energy because it is governed by the RS mechanism. The other peculiarity is that in the low energy excitation case, the "reduced" light sums dominate even more over the "increased" blue light sums than at $\lambda_{exc} = 320$ nm.

Qualitatively, the same observations as for serum we have done with urines but the spectral effects are noticeably weaker in this case. For HDs these measurements were not performed because of their strong initial dilution.

5.3. *Manifestation of VF concentration self-quenching at different detection coordinates*

The form of concentration quenching curves depends very much on detection coordinates in excitation/emission matrixes. We have observed at the higher exc/em energies, e.g., at $\lambda_{exc} \leq 325$ nm and $\lambda_{em} < 430$ nm the fluorescence intensity increases in some urines in the dilution range 100% → 65%, i.e., when VFS concentration decreased. In Fig. 5, a curve of such type is depicted for $\lambda_{exc}/\lambda_{em} = 315/410$ nm. Seemingly, this a manifestation of Stern–Volmer's self-quenching in auto-fluorescence of biofluids.

In the measurements with lower exc/em energies that we have observed for the same samples, only intensity decreases by dilution in both serum and urine. The particular observation result at a constant λ_{exc} depends strongly on the emission wavelength chosen for detection. In Fig. 5, are depicted two curves for $\lambda_{em} = 400$ and 560 nm obtained at $\lambda_{exc} = 360$ nm. We see a tendency to intensity saturation for both curves but the slopes are remarkably different as one can conclude from polynomial equations for these curves (curves are normalized at the concentration point 40%). By decrease of VFS concentration, the emission at 560 nm is relatively more intensive but decreases at a higher speed than the emission at 400 nm. Qualitatively the same difference can be observed for all possible combinations of exc/em energies in urine and in serum as well. Than at the longer wavelengths the measurement is being done so the bigger is the difference in relation, e.g., to the level with $\lambda_{em} = 400$ nm which is taken for comparison purposes. Additionally, for a fixed λ_{em}, similar effect can be observed for increasing λ_{exc} if the corrections to the absorption level are done.

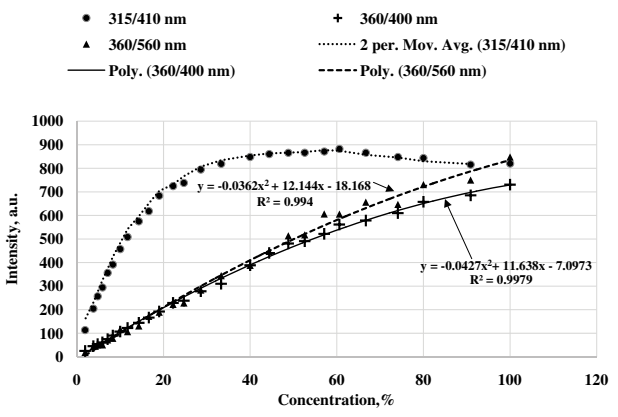

Fig. 5. Fluorescence intensity dependences in a Pt's urine (protein-uria) on the VFS residual concentration at the different parameters of excitation and emission registration.

Most probably this effect is induced by different number of destroyed/created fluorescence centers of a certain profile during dilution/enhancing concentration of VFS. We think that conceivable VF quantum yield changes and modified interactions with the main related constituents in urine and serum in the course of dilution have only minor effect if at all. We did not find any remarkable influence on VF intensity when tentatively enhanced the concentration of urea, creatinine, uric acid, ions of Na, Ca, ammonia chloride. So, this is one more manifestation of multiparticle association/dissociation effects and modification of VFS itself in the course of its concentration change without chemical transformations.

Whatever the mechanisms affecting the VF intensity are, it is clear that nonlinearities observed here greatly limit the use of VF in serum and urine for diagnostics or monitoring.

5.4. *VF registration in HDs as a suitable tool for dialysis monitoring*

On the contrary to urine and serum, we did not encounter nonlinearity effects in VF of all HDs by almost all excitation/emission combinations. Only for $\lambda_{exc} < 315$ nm, weak influence of the UV emission tail (see Fig. 2) rarely was observed and the linearity could be disturbed. In most cases, the dependences "VF intensity versus dilution" were strictly linear and we have made use of these favorable features as it follows.

For online monitoring of hemodialysis procedure among the other methods, those based on the UV absorption measurements ($\lambda \approx 285$ nm) are being

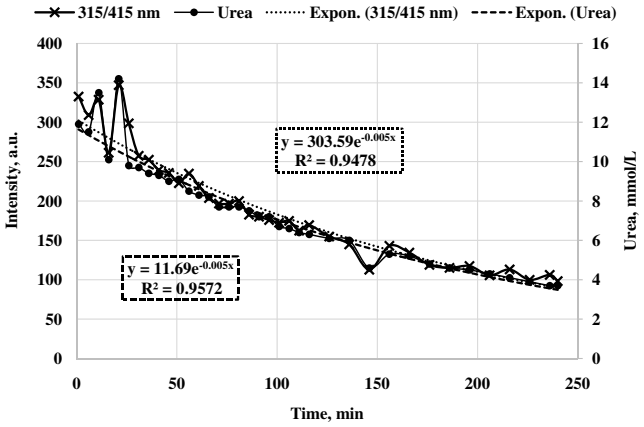

Fig. 6. Time evolution of a dialysis procedure at a Fresenius 5008 CorDiax machine (filter FX 100). Fluorescence was measured offline at $\lambda_{exc}/\lambda_{em} = 315/415$ nm.

applied nowadays.[16,17] Additionally, the visible (456 nm) and near UV (358 nm) fluorescence signals were proposed to control indirectly the extraction of Beta2-microglobulin and indoxyl sulphate specifically.[18,19] Monitoring of the abundant metabolite-urea extraction by fluorescence method was not proposed yet.

We have collected samples of spent dialysate in 5–10 min intervals during hemodialysis procedures in the TUH. One part of samples was given immediately to the laboratory for biochemical assays of urea, uric acid, creatinine, phosphates, residual proteins, etc. With the second part of samples, the VF measurements were done in approximately 2 h after the start and end of HD procedures of ≈ 4 h duration at the most.

In Fig. 6, one can see how the urea concentration and VF intensity during the HD procedure are changed in the spent dialysate. We see that coincidence of exponential temporal curves is good. Clearance indexes $Kt/V = 1.17$ (urea) and 1.22 (VF) obtained for both curves lay fairly in the desired range of 1.20 ± 0.20.[17] The same result was obtained for creatinine extraction.

This pilot experiments show that there are prospects to elaborate the VF measurements method for online monitoring of hemodialysis procedures.

6. Discussion

Nonlinear effects and intensity spectral mirror-reversed redistribution in VF of biofluids that we have observed by dilution of serum and urine can be, to our opinion, most straightforwardly explained by association/dissociation of the constituent non-protein molecules. The phenomenon of binding of proteins and small molecules to proteins is well known and is one of the important aspects in the study and monitoring of HD procedures.[17,20] There were discovered and investigated (by methods other than fluorescence) the nanoentities (nanoassociates, supramolecules, loose nanoassociates, etc.) dissolved in water.[21–24] Some of substances used in the experiments[21–24] are of great biological importance — urea, glucose, acetic acid, sodium chloride. These substances are not fluorophores, however. Therefore, we assume further that the association can also be a characteristic for the small molecules which can fluoresce. So far, they remain mostly unidentified in biofluids and cannot be addressed unambiguously (see also below).

Earlier[5,6] we found a plenty (>10) of strong similarities in optical-fluorescent properties of biofluids and carbon-based nanoparticles (CNP) aqueous solutions. By the dynamic light scattering (DLS) and small angle X-ray scattering (SAXS) methods the smallest nanoparticles of the diameter 5–7 nm in protein-free fractions of urine were detected. Recently, we got images in a scanning electron microscopy (SEM) of dry protein-free urine fractions showing nanoparticles and their aggregates on the gold substrates with individual diameters expanding from ~ 5 up to ~ 100 nm.[25] Elemental analysis performed in SEM (*in situ*) has shown that these nanoparticles are rich in carbon and oxygen.

The results obtained here by dilution at usual environmental conditions point to the association binding as the most probable driving mechanism of self-assembly of catabolic molecules in biofluids. The vast majority of studies of molecular association in liquids show, however, that there are few common physical laws and norms which fully govern the association processes. As a result, almost all types of molecules need individual and scrupulous approach and investigation of their association.[21–24,26] Concentration of dissolved substances, time intervals needed for completion of association vary in the huge range of many orders, e.g., from the macrolevel of tens wt.% down to the very low 10^{-20} mol/L.[21–24] Measured formation time intervals are also reported to be very different — from several seconds ("almost immediately"[21–23]) up to the weeks.[21–24] Dimensions of nanodomains vary in their three orders of magnitude from several up to hundreds of nanometers. The glycerol domain formation

in water occurred after some calm delay in a very abrupt manner.[21–23] Environmental magnetic fields strongly stimulate creation of nanoassociates.[24]

Presumably, in biofluids we have a kind of catabolic molecules fast to associate. The filtration of blood in the kidneys or in dialysis machines prevents the appearance of large particles ($d > 10$ nm) in urine and HDs if such "giants" do not arise in these biofluids immediately after filtration. We have observed sometimes in HDs VF with exclusively high intensity, what can mean that some critical condition(s) was (were) spontaneously met for VFS nucleation (maybe, similarly to the glycerol case).

Usually, we did not observe remarkable, i.e., more than 10%, changes in emission intensity during several hours after sampling of our biofluids. The degradation of real biofluids at room temperatures prevented the temporal experiments with large duration in our case (usually the duration of our experiments was 3 h or less). The dilution experiments did not show any delays or hysteresis like behavior in the scales "dilution-VF intensity", what also supports the hypothesis about fast occurrence of association/dissociation processes.

To identify the concrete catabolic molecule(s) giving VF in biofluids, we have started with characteristic reaction of aluminium ions Al^{3+} in biofluids.[6] We have found that it is unlikely that this substance is xanthine or 3-hydroxyanthranilic acid. Xanthine presence is well established in HDs[20] and assumed to be the case in urine.[14] Both metabolites exhibit quite intense fluorescence with $\lambda_{excmax}/\lambda_{emmax} \approx 320/420$–$430$ nm. There are in biofluids other small molecules with fluorescence in the blue (visible) spectral region[27] which must be checked too and therefore our study continues. For instance, there is a lot of information about the heterogeneous superclass of advanced glycation end-products (AGE) present in human biofluids (see, e.g., review Ref. 28). The AGE family components are mostly not yet identified. An exclusion makes only the fluorescent pentosidine $C_{17}H_{26}N_6O_4$ ($M = 378$, 4 Da) with $\lambda_{excmax}/\lambda_{emmax} = 335/385$ nm. The dominant mass distribution of fluorescent AGEs is in the range $1 < M < 3$ kDa. This fact may indicate not permanent multicomponent architecture of fluorescent AGE moieties rather than its rigid single molecules character.

The properties of the group of biological substances with blue (visible) emission can be compared with those of oxidized fluorescent graphene ($\lambda_{excmax}/\lambda_{emmax} \approx 320/420$ nm).[5,29] Graphene is an interesting example as a nitrogen-free compound what makes it close to ninhydrin-negative VFS.[3] Our preliminary matrix assisted laser dissociation/ionization (MALDI) experiments (will be described elsewhere) have shown the appearance of typical ionic fragments of masses 213 ± 1 and 413 Da in the spectra of both graphene and urine fraction 1. Such parallel appearance creates strong doubt that ion $M = 213$ Da is indoxyl sulfate $C_8H_7NO_4S$ ($M = 213.2$ Da) which is typically present in biofluids. Strongly hydrophilic VFS[3] can belong to the class of substances with fused aromatic rings and numerous bound OH ions. The fractions 1 of HD have always higher pH values than the whole HDs, i.e., ≈ 8.5 and ≈ 7.5, accordingly. Chemical superclasses of aromatic heteromono- heteropoly-, homomono- and homopolycyclic compounds count, respectively, $67 + 728 + 432 + 6 = 1233$ items and constitute the most numerous ensemble in the urine metabolome database.[27] Some metabolites from this database are food derivatives.

There is one more feature in our numerous experiments with HDs (more than 200 VF measurements): the mortality among CKD Pts is higher for individuals with higher VF intensity. The statistics is small (7 people dead), however, it should not be neglected.

7. Conclusion

The dilution experiments with serum and urine have shown that in these biofluids the intensity of visible auto-fluorescence is always suppressed by concentration quenching. In sera, the effect is weaker and manifests itself only in sublinear dependence of intensity versus concentration, whereas urine quenching is usually stronger and can be seen as fall in absolute intensity values (up to 20% in relation to maximum) in some undiluted samples. This nonlinearity restricts the use of VF intensity as a marker for these two biofluids. At the same time for HDs, which are similar to strongly diluted urines, the linearity "VF intensity–VFS concentration" is always observed. The VF intensity in HDs can serve as a biomarker for photonics-based monitoring of hemodialysis, what was demonstrated in a special case of simultaneous offline monitoring of extraction of urea and other metabolites in the real hemodialysis procedures.

Nontrivial results have been obtained in the form of mirror-reversed spectral redistribution of VF intensity as a result of simple dilution of serum and urine samples. During dilution, the intensity in the long wavelength part of VF around 500 nm decreases whereas the intensity in the region around 400 nm increases. The phenomenon can be interpreted as a manifestation of self-assembling of small fluorescent moieties (maybe, separate molecules, dimers, trimers, etc.) into larger nanoparticles when their concentration increases in undiluted serum and urine. Taking into account the earlier revealed capability of such biologically important molecules as urea, sodium chloride, glucose etc. to associate into nano domains[20–23] one can conceive that the non-protein VFS in biofluids *in vivo* is also in nanoassociate condition. Together with the overall negative context of the presence of VFS in biofluids, the matter can be interpreted as endogenous nanotoxicology. Such a consideration would be a new approach to some pathological processes in human body.

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