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Structural Typing of Systemic Amyloidoses by Luminescent-Conjugated Polymer Spectroscopy

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Most systemic amyloidoses are progressive and lethal, and their therapy depends on the identification of the offending proteins. Here we report that luminescent-conjugated thiophene polymers (LCP) sensitively detect amyloid deposits. The heterodisperse polythiophene acetic acid derivatives, polythiophene acetic acid (PTAA) and trimeric PTAA, emitted yellow-red fluorescence on binding to amyloid deposits, whereas chemically homogeneous pentameric formic thiophene acetic acid emitted green-yellow fluorescence. The geometry of LCPs modulates the spectral composition of the emitted light, thereby reporting ligand-induced steric changes. Accordingly, a screen of PTAA-stained amyloid deposits in histological tissue arrays revealed striking spectral differences between specimens. Blinded cluster assignments of spectral profiles of tissue samples from 108 tissue samples derived from 96 patients identified three nonoverlapping classes, which were found to match AA, AL, and ATTR immunotyping. We conclude that LCP spectroscopy is a sensitive and powerful tool for identifying and characterizing amyloid deposits. (*Am J Pathol* 2010, 176:563–574; DOI: 10.2353/ajpath.2010.080797)

Amyloidoses are diseases of disparate etiologies characterized by extracellular proteinaceous deposits in tissues and organs. These deposits, termed “amyloids,” result from misfolding and/or partial unfolding of proteins followed by their ordered aggregation. At least 27 different proteins have been reported to form disease-associated amyloids *in vivo*¹, and there is evidence that any

given polypeptide can be induced to form amyloid *in vitro* under appropriate conditions.² It has been suggested that amyloid may represent a primordial state for all proteins, which would be always attained unless specific antiamyloidogenic factors (including chaperones and disaggregases) prevent or revert its formation. Accordingly, formation of amyloids may be much more prevalent than currently appreciated³ and may contribute to several diseases whose etiology is hitherto unclear.⁴

Some amyloids are highly selective in their organ tropism and lead to localized amyloidosis. Two highly prevalent organ-specific amyloidoses are caused by the A β peptide⁵ and islet amyloid polypeptide, which are associated with Alzheimer's disease and type 2 diabetes, respectively. Prion diseases, which go along with aggregation of PrP^{Sc}, can be regarded as transmissible amyloidoses of the nervous and lymphoreticular system.⁶ Other amyloidogenic proteins deposit in many organs. Such “systemic” amyloidoses can be neoplastic, inflammatory, or genetic in origin and present as chronic progressive diseases leading to lethal heart and renal failure if untreated. Depending on the biochemical nature of the amyloid in question, the average survival can be as short as 12 months, or can span 7–15 years.

Therapeutic options for amyloidoses include aggressive treatments directed at reducing precursor protein production and/or at inhibiting the extracellular deposi-

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tion of amyloid fibrils, eg, by pharmacological immunosuppression or even by surgical replacement of the organ synthesizing the precursor protein. The indication for each of these therapies, and their prognostic prospect, depend on the specific type of amyloid.^{7,8} As optimal outcomes rely on early therapy, sensitive methods for diagnosis and selective technologies for identification of amyloid subtypes are of greatest importance.

Systemic amyloidoses are classified according to the chemical nature of the predominant amyloid constituent, with the most common amyloids being AL, AA, and ATTR.^{9,10} AL amyloid consists of Ig light chains, or fragments thereof, and is associated with various B cell lymphoproliferative disorders including multiple myeloma.^{10–13} AA amyloid is derived from serum amyloid A (AA) protein and is associated with chronic immune activation, as in chronic infections, autoimmune or hereditary inflammatory diseases, or cancer.^{14,15} ATTR systemic amyloid consists of transthyretin deposits and occurs sporadically or in association with transthyretin mutations that enhance protein misfolding and fibril formation.^{16–19}

Amyloid deposits consist of fibrils of 7–10 nm in diameter displaying a cross β -pleated sheet conformation. This common structural property has enabled the development of generic amyloid ligands, such as Congo Red and thioflavins. The gold-green birefringence of Congo Red-stained amyloid is commonly considered the diagnostic gold standard. However, small amyloid ligands do not differentiate between amyloid subtypes. Hence, the biochemical classification of amyloids must be pursued by other means.

In hereditary amyloidoses, certainty can be attained by identifying causative mutations in the genes encoding the respective precursor protein. Mass spectrometric identification of amyloid-derived peptides can be diagnostic, but it is far too cumbersome for routine clinical diagnostics. Therefore, immunohistochemical stains are typically used for differentiating amyloid subtypes.¹³ However, immunohistochemistry is fraught with specific problems. Most antibodies penetrate only poorly the compact amyloid structures. Also, many amyloids incorporate Igs and complement-derived opsonins, which can give rise to false-positive stains with diagnostic antibodies and—in worst-case scenarios—may lead to misdiagnoses.^{13,20–23}

We have previously reported that luminescent-conjugated polymers (LCPs) interact with amyloid fibrils and amyloid deposits in tissue sections.^{24–26} Whereas conventional amyloid ligands, such as Congo Red and thioflavin derivatives, are sterically rigid and fluoresce with defined spectra, LCPs are composed of rotationally flexible polythiophene chains that fluoresce in different colors depending on their geometry. Therefore, the emission spectra recorded from LCPs reflect the conformation of their backbones. The interaction of LCPs with protein aggregates imposes rotational constraints leading to spectroscopic signatures indicative of specific supramolecular structures. This phenomenon allows for discriminating mouse-passaged prion strains that may solely differ in their structure.²⁷ Analogously, we were able to

differentiate multiple heterogeneous types of A β deposits in the brain of transgenic mice.²⁸

Here we tested the usefulness of three anionic LCPs, polythiophene acetic acid (PTAA), trimeric polythiophene acetic acid (tPTAA), and pentameric formic thiophene acetic acid (p-FTAA) for the typing of human systemic amyloidoses. We found that the analysis of spectral signatures of anionic LCPs provide complementary information to conventional techniques regarding the nature of the amyloid deposits and that LCP fluorescence provides a more precise readout than the polarized absorbance of Congo Red. Hence, LCPs represent a complementary tool for rapid and accurate diagnosis of systemic amyloidoses.

Materials and Methods

Patients

Patients diagnosed with amyloidosis during the period from 1996 to 2007 were identified in the archives of the Institute of Surgical Pathology (Zurich, Switzerland). We chose 54 paraffin-embedded tissue blocks with conspicuous amyloid deposits from 42 patients, including 10 women and 32 men. Clinical information was retrieved from hospital records. The mean age at diagnosis was 70.5 years (range, 17–96 years). The tissue samples of the 42 patients with systemic amyloidosis included heart ($n = 27$), kidney ($n = 4$), tongue ($n = 1$), esophagus ($n = 1$), seminal vesicle ($n = 1$), stomach ($n = 2$), soft tissue ($n = 2$), lymph node ($n = 1$), lung ($n = 2$), sigma colon ($n = 1$), small intestine ($n = 1$), duodenum ($n = 2$), conjunctiva ($n = 1$), liver ($n = 1$), colon ($n = 1$), prostate ($n = 1$), thyroid ($n = 1$), adrenal gland ($n = 1$), parathyroid ($n = 1$), and joint capsule ($n = 2$). All tissue specimens were fixed in 4% formalin and embedded in paraffin. The present study was performed according to the ethical rules for establishments of novel diagnostic tests in the Kanton Zurich under strictly unlinked-anonymous conditions.

A “validation set” of amyloid specimens was retrieved from the Amyloid Registry of the Charité University Hospital (Berlin, Germany) and included 54 patients with histologically confirmed amyloid (37 males, 17 females; mean age, 66.4 years; range, 42–86 years). The tissue samples were obtained from the heart (17 cases), colon and rectum (12), stomach (5), liver (4), kidney (3), iliac crest (3), lung (2), skin (2), lymph node (1), small intestine (1), tendons (1), thyroid gland (1), tongue (1), and ureter (1). All specimens had been fixed in formalin and embedded in paraffin.

Histology and Immunohistochemistry

In each sample selected for this study, the presence of amyloid was confirmed by histological examination of the birefringence of deparaffinized, Congo Red-stained sections. The quality of the tissue material and amount of amyloid deposits were documented for each sample. Amyloid subtypes were classified by immunohistochem-

istry with a broad panel of reagents including commercially available primary monoclonal or polyclonal antibodies. Antibodies against AA (diluted 1/500; monoclonal), transthyretin (TTR) (1/800; polyclonal), human kappa light chains (κ ; polyclonal), and lambda light chains (λ ; polyclonal) were purchased from Dako (Glostrup, Denmark). Primary monoclonal antibodies against human kappa (κ) and lambda (λ) light chains (1/50) were purchased from BMA Biomedicals (Augst, Switzerland). Staining of the tissue microarrays (TMAs) was performed at the Institute of Pathology of the Basel University Hospital (Basel, Switzerland). Immunohistochemistry was performed on deparaffinized formalin-fixed tissue using an automated staining system (Ventana Medical Systems, Tucson, AZ) or manually. Slides were heated with cell conditioner for antigen retrieval and endogenous biotin was blocked with a standard kit. Immunoreactivity was visualized with iVIEW DAB detection kit. Slides were counterstained with hematoxylin before glass coverslipping, and interpreted according to recent recommendations.²⁹ The amyloid deposits of the "validation set" were classified immunohistochemically as described elsewhere.³⁰

LCP Synthesis and LCP Staining

The synthesis of PTAA (mean mol. wt., mol. wt. = 3 kDa), tPTAA (mol. wt. = 1.5 kDa) and p-FTAA (mol. wt. = 615 Da) have been reported elsewhere.^{31–33} Paraffin-embedded formalin fixed sections were deparaffinized. After rehydration with deionized water, the sections were equilibrated in PBS (p-FTAA staining) or 100 mmol/L sodium carbonate at pH = 10 (PTAA and tPTAA staining). LCPs were diluted in incubation buffer (10 μ g/ml), added to the tissue sections, incubated for 30 minutes at room temperature, and removed by washes with incubation buffer.

Fluorescence Microscopy and Spectral Analysis

Images and spectra were recorded with a Zeiss Axioplan 2 microscope fitted with a Spectraview 4.0 (Applied Spectral Imaging, Migdal, Israel) and a Spectra-Cube (interferometrical optical head SD 300) module with cooled CCD-camera, through a 405/30-nm (LP 450) bandpass filter. The data were processed with Spectra-View 3.0 EXPO software. Spectra were collected from LCP stained amyloid deposits (5–10 deposits for each sample and eight individual spots from each deposit) and other fluorescent entities. Fluorescent spectral unmixing (SUN) was performed using the function in the software. The spectra recorded for amyloid deposits in positive samples were used for SUN analysis of the negative control sample.

Construction of Amyloid TMAs

An amyloid TMA was constructed to investigate the LCP selectivity for amyloid. The TMA approach allows the direct comparison between many different subtypes of amyloids and negative controls within one small tissue

cylinder. Paraffin-embedded tissues were selected for the TMA construction on the basis of Congo Red-stained tissue sections. Only tissue blocks with conspicuous amyloid deposits were used. Up to four cores (0.6 mm in diameter; length: 3–4 mm) were taken from each representative tissue block to increase the possibility of sufficient amyloid deposits in the tissue cores of the TMA. Cores from four different tissues without amyloidosis (heart, pancreas, spleen, and kidney) were included as negative control. The cores were transferred to the recipient paraffin block as described previously.³⁴ Four-micrometer-thick sections were cut for further analysis. There were sufficient amyloid deposits with positivity for both Congo Red and immunohistochemistry in tissue cores of 20 patients.

Statistical Methods

For spectral collection of PTAA bound to amyloid aggregates, tissue sections were analyzed as follows: eight individual spots within each of 5–10 plaques from each case were examined, yielding 30–50 measurements per case. The fluorescent intensity ratios were calculated ($R_{538\text{ nm}/E_{\text{max}}}$ and $R_{538\text{ nm}/642\text{ nm}}$), and mean and SD were recorded for each spectral ratio for each individual. To make up homogeneous groups of objects (classes) on the basis of their description by the two ratios, a k-means clustering analysis was performed (XLSTAT, 2008). In addition an unpaired, two-tailed Student's *t*-test was performed using mean values of single cases as observations (GraphPad Prism 5). The raw data from each individual case and the results from the statistical evaluations are provided as Supplemental Tables S1–S14 at <http://ajp.amjpathol.org>.

Results

Immunohistochemical Amyloid Classification

Amyloid deposits in 54 tissue samples from 42 patients were typed by immunohistochemistry on TMAs and on selected conventional tissue sections (Tables 1 and 2). For the nomenclature of amyloid and amyloidoses, we followed the recommendations by the International Nomenclature Committee on Amyloidosis. We stained TMA sections with AA, AL, or ATTR antibodies and scored immunohistochemical signals as interpretable only when they colocalized with unambiguous Congo Red signals.

When performing immunostains with antibodies to AL, we detected weak signals in all amyloid deposits. This is consistent with the reports that Ig λ -light chains frequently contaminates amyloid deposits of all types and can give rise to misinterpretations.^{13,20–23} Therefore, AL-positive samples were classified as AL amyloid only if they were negative for all other immunohistochemical amyloid stains (AA and ATTR). All other sections were initially stained with the AA antibody. If the samples were negative for AA, immunohistochemistry for ATTR and/or AL (m- κ , p- κ , m- λ , and p- λ) was performed.

Table 1. Clinical Description and Tintorial Properties of TMA Specimens

Patient	Organ	Primary diagnosis	Age/Sex	Congo Red*	IHC [†]	PTAA [‡]	tPTAA*	p-FTAA*
1	Heart	Heart failure	80/M	+	ATTR	1	+	+
2	Heart	Lung carcinoma, atrial fibrillation	85/M	+	ATTR	1	+	+
3	Heart	Trauma	84/M	+	ATTR	1	+	+
4	Heart	Hepatocellular carcinoma, hypertension	80/M	+	ATTR	1	+	+
5	Heart	Hypertensive heart disease	90/M	+	ATTR	1	+	+
6	Heart	Myocardial amyloidosis, atrial fibrillation	96/M	+	ATTR	1	+	+
7	Heart	Chronic pneumonia	85/F	+	ATTR	1	+	+
8	Esophagus	Lung and prostate carcinoma, rheumatoid arthritis	77/M	+	AA	2, 3	+	+
8	Kidney	Lung and prostate carcinoma, rheumatoid arthritis	77/M	+	AA	2, 3	+	+
9	Heart	Hypertensive heart disease	82/M	+	ATTR	1	+	+
10	Heart	Cardiomyopathy	85/M	+	ATTR	1	+	+
11	Heart	Primary amyloidosis	84/F	+	AL	2	+	+
12	Heart	Multiple myeloma	75/F	+	AL	2	+	+
12	Tongue	Multiple myeloma	75/F	+	AL	2	+	+
13	Heart	Ileal adenocarcinoma	75/F	+	ATTR	1	+	+
14	Kidney	Monoclonal gammopathy	73/F	+	AL	2	+	+
15	Seminal vesicle	Lung carcinoma	73/M	+	AL	2	+	+
16	Heart	Cardiomyopathy	88/M	+	ATTR	1	+	+
17	Heart	Amyloidosis, myocardial infarction	95/M	+	ATTR	1	+	+
18	Heart	Coronary heart disease, suspected amyloidosis	76/M	+	ATTR	1	+	+
19	Stomach	Abdominal pain	71/M	+	AL	2	+	+
20	Heart	Suspected amyloidosis	79/M	+	ATTR	1	+	+
21	Heart	Trauma	17/F	—	—	—	—	—
21	Pancreas	Trauma	17/F	—	—	—	—	—
21	Spleen	Trauma	17/F	—	—	—	—	—
21	Kidney	Trauma	17/F	—	—	—	—	—

*Positive or negative staining are represented with + or —, respectively.

[†]Amyloid subtype according to immunohistochemistry (IHC).

[‡]The numbers 1, 2, or 3 refer to the spectral classification of PTAA tingibility.

M, male; F, female.

Of the 42 patients with amyloidosis studied here, immunohistochemical typing determined that 18 were ATTR, 18 were AL, and 6 were AA (Tables 1 and 2). The plausibility of immunohistochemical typing was corroborated by the evaluation of clinical diagnoses. For negative controls, we used 11 tissue samples from three patients with neoplastic diseases (multiple myeloma without amyloidosis; lung cancer), two patients with chronic inflammatory disorders (chronic obstructive pulmonary disease; gonarthrosis), one patient with lysosomal storage disease (Fabry's disease), and one young patient who died as a result of trauma and was devoid of any systemic or organ-specific amyloid (Tables 1 and 2).

Selective Amyloid Detection by LCP

In a first series of experiments, the sensitivity and selectivity of LCPs were tested with an amyloid TMA containing samples from 20 amyloidosis patients and one negative control. PTAA, tPTAA, and p-FTAA (Figure 1, A–C) identified all amyloid deposits that were stained by antibodies and Congo Red, whereas none of the negative control samples showed any LCP signal (Figure 2, A–D). Amyloid deposits stained with PTAA or tPTAA emitted yellow-red fluorescence, whereas p-FTAA emitted extremely intense green-yellow fluorescence.

We then recorded full visible-light emission spectra (450–700 nm) on excitation through a 405/30 long-pass

filter (LP 450) from LCP-stained amyloid deposits with a Spectramax camera at intervals of 8–10 nm (Figure 3, A–D). These measurements confirmed that p-FTAA had its absolute emission maximum at a wavelength of ~550 nm, whereas the emission maxima for PTAA and tPTAA were located at longer wavelengths (560–600 nm), thereby conferring a reddish hue to the emitted light. These spectral characteristics were used to deconvolute the signals obtained from the specimens by “SUN”: those pixels in each region of interest that displayed an LCP characteristic emission spectrum were arbitrarily assigned to red (PTAA and tPTAA) or green (p-FTAA) pseudocolor codes, whereas fluorescent structures with other emission spectra were not visualized (Figure 2).

We directly compared sequential, adjacent sections stained with Congo Red and examined for green birefringence with those stained with LCPs. We found it much easier to identify amyloid deposits using the LCP fluorescence. Owing to its very high quantum yield, p-FTAA proved to be the most sensitive dye. Whereas collagen typically gave rise to some confounding birefringence in negative control samples (Figure 2), collagen did not score positive with any of the LCP stains. Even if not expressed in strictly quantitative terms, these results indicate that LCP fluorescence was vastly more sensitive and displayed much better signal-to-noise ratios than inspection of Congo Red-stained sections under cross-polarized light.

Table 2. Clinical Description and Tinctorial Properties of Additional Samples

Patient	Organ	Clinical information	Age/Sex	Congo Red*	IHC†	PTAA group‡
8	Heart	Lung and prostate carcinoma, rheumatoid arthritis	77/M	+	AA	2, 3
8	Lung	Lung and prostate carcinoma, rheumatoid arthritis	77/M	+++	AA	2, 3
8	Liver	Lung and prostate carcinoma, rheumatoid arthritis	77/M	++	AA	2, 3
8	Colon	Lung and prostate carcinoma, rheumatoid arthritis	77/M	++	AA	2, 3
8	Prostate	Lung and prostate carcinoma, rheumatoid arthritis	77/M	++	AA	2, 3
8	Thyroid	Lung and prostate carcinoma, rheumatoid arthritis	77/M	+++	AA	2, 3
8	Adrenal gland	Lung and prostate carcinoma, rheumatoid arthritis	77/M	+++	AA	2, 3
8	Parathyroid	Lung and prostate carcinoma, rheumatoid arthritis	77/M	+++	AA	2, 3
12	Lung	Multiple myeloma	75/F	+++	AL	2
22	Lymph node	Lymphadenopathy	49/F	+++	AA	3
23	Kidney	Proteinuria, suspected amyloidosis	36/F	+	AA	3
24	Kidney	Proteinuria	17/M	+	AA	3
25	Small intestine	Diarrhea, nephrotic syndrome	38/M	+++	AA	3
25	Stomach	Diarrhea, nephrotic syndrome	38/M	+++	AA	3
26	Duodenum	Duodenal ulcer	53/M	++	AL	2
27	Duodenum	Suspected amyloid	47/M	++	AL	2
28	Sigma colon	AL amyloidosis	39/F	+	AL	2
29	Heart	Suspected amyloidosis	74/M	+++	ATTR	1
30	Heart	Suspected cardiomyopathy	62/M	++	AL	2
31	Heart	Heart failure, monoclonal gammopathy	80/M	++	ATTR	1
32	Heart	Coronary heart disease, suspected AL amyloidosis	87/M	++	ATTR	1
33	Heart	Proteinuria, diarrhea, arrhythmia	77/M	+++	AL	2
34	Heart	Multiple myeloma	61/F	++	AL	2
35	Heart	Multiple myeloma	60/F	+++	ATTR	1
36	Heart	Multiple myeloma	68/M	++	AL	2
37	Heart	Multiple myeloma	90/M	+	AL	2
38	Heart	Multiple myeloma, nephrotic syndrome	54/M	++	AL	2
39	Joint capsule	Fibrosis	67/M	+++	AA	3
40	Soft tissue	Amyloidosis	71/M	++	AL	2
41	Joint capsule	Arthritis	70/M	++	AL	2
42	Conjunctiva	Smoldering lymphoma with AL amyloidosis	63/M	+++	AL	2
43	Soft tissue	Suspected Ganglion, amyloid	64/M	+++	AL	2
44	Heart	Multiple myeloma, heart disease	57/M	—	Neg	Neg
45	Heart	Multiple myeloma, heart failure	31/M	—	Neg	Neg
46	Joint capsule	Gonarthrosis	74/F	—	Neg	Neg
47	Lung	Chronic obstructive pulmonary disease	64/F	—	Neg	Neg
48	Lung	Suspected malignancy	62/F	—	Neg	Neg
49	Stomach	Fabry's disease	17/M	—	Neg	Neg
49	Duodenum	Fabry's disease	17/M	—	Neg	Neg

*Positive or negative staining are represented with + or —, respectively.
†Amyloid subtype according to immunohistochemistry (IHC).
‡The numbers 1, 2, or 3 refer to the spectral classification of PTAA tingibility.
M, male; F, female.

Spectral Assignment of Amyloid Deposits

When examining samples stained with PTAA and excited with a 405/30 nm (LP 450) bandpass filter, we found obvious interindividual variation in the color of light emit-

Figure 1. Chemical structures of the LCPs used in this study. PTAA (A); trimeric PTAA, tPTAA (B); and p-FTAA, pentameric formic thiophene acetic acid (C). The number of monomeric units (n) for the polydispersed LCPs was typically between 11 and 20 (PTAA) or 3 and 4 (tPTAA). All LCPs were synthesized as sodium salts, and all displayed anionic net charges under the staining conditions used in this study.

ted by amyloid deposits. The formal analysis of their emission spectra revealed emission maxima for each deposit at either of the three wavelengths, 570, 580, and 590 nm (Figure 3). These signatures are known to reflect specific geometries of the LCP chains. Nonplanar, disperse LCP chains emit light $\sim 530-540$ nm, whereas planar transition of the backbones shifts their emission maximum toward longer wavelengths. This transition is often accompanied by stacking of the LCP chains, leading to intermolecular fluorescence resonance energy transfer and to a relative increase in emission intensity at $\sim 640-650$ nm.³⁵⁻³⁷ Therefore, we used the ratios of the intensity of the light emitted at the above wavelengths, $R_{538/E_{max}}$ and $R_{538/642}$, as spectral surrogates for the conformation of amyloid-bound PTAA (Supplemental Tables S1 and S2, see <http://ajp.amjpathol.org>).

When plotting the values of the $R_{538/E_{max}}$ and $R_{538/642}$ ratios for each of the amyloid deposit from the 42 patients, we found that each sample segregated into one of three sharply demarcated groups (Figure 3; details of the k-mean clustering analysis are reported

Figure 2. Immunohistochemical stains, Congo Red (CR) polarization microscopy, and LCP histochemistry of selected TMA spots. Each panel (A–D) depicts serial TMA sections stained with H&E, antibodies to the relevant amyloid constituents (ATTR, AA, AL, B2M), and LCPs as indicated. The **middle columns** depict original fluorescent micrographs, whereas the panels on the right represent pseudocolor visualizations after SUN: PTAA and tPTAA signals are represented in red and pFTAA staining in green. **A:** Kidney sample of patient 8 who had been diagnosed with AA amyloidosis. **B:** Heart sample of patient 6 diagnosed with ATTR amyloidosis. **C:** Seminal vesicle sample of patient 15 diagnosed with AL. **D:** Heart specimen of patient 21 who did not suffer from amyloidosis (negative control). The silvery birefringent material in the CR stain consists of collagen fibers; this material does yield any signal in the LCP stains. Scale bars: 250 μ m.

as Supplemental Tables S3–S8, see <http://ajp.amjpathol.org>). Similar but less dramatic spectral changes were observed for tPTAA, whereas the p-FTAA spectrum did not appreciably change on binding to the various amyloids (Figure 3).

We next compared the spectral data of PTAA with the immunohistochemical results. The spectral classification of all of the samples correlated with the amyloid type according to the immunohistochemical classification with PTAA group 1 for ATTR, PTAA group 2 for AL, and PTAA

Figure 3. Characterization of the fluorescence emission of LCP-stained amyloid deposits. **A–C:** Representative spectra from amyloid deposits stained with PTAA (**A**), tPTAA (**B**), or p-FTAA (**C**). For PTAA, spectra with emission maxima at 570 (blue), 580 (green), or 590 nm (red) was observed. On staining with tPTAA and p-FTAA, differences between spectra were much more modest. Spectra were selected from samples (triangles) belonging to each of the three distinct groups observed in the correlation diagram of the spectral ratios. The wavelengths used for calculation of the spectral ratios $R_{538/Emax}$ and $R_{538/642}$; 538 nm (*), Emax (+) and 642 nm (–), respectively, are highlighted. **D:** Correlation diagram of the spectral ratios, $R_{538/Emax}$ and $R_{538/642}$, for PTAA from all cases that were stainable with CR. The mean value of eight spots from 5 to 10 individual amyloid deposits in each tissue section is represented as a square. The samples segregated into three nonoverlapping groups. Comparisons to immunohistochemical results revealed that these groups corresponded to AA (blue squares), AL (green squares), and ATTR (red squares) amyloidosis. The insert picture is showing a zoom in of the group corresponding to ATTR amyloidosis. The mean value and the SD for each group are shown as circles with black error bars. For statistical details and data for individual samples see Supplementary Tables S1–S8 at <http://ajp.amjpathol.org>.

group 3 for AA (Tables 1 and 2). Hence, the emission profiles of PTAA appear to reliably distinguish between amyloids of distinct biochemical subtypes. In one of the cases (patient 8), we found amyloid having two distinct PTAA spectra corresponding to groups 2 and 3, whereas the immunohistochemical analysis only revealed AA staining (Figure 4, A–F). Intriguingly, 10 different organs from this case were analyzed and all of them contained amyloid with two distinct PTAA spectra (Tables 1 and 2).

Validation of the Spectral Assignment

In a second set of experiments, the PTAA spectral classification was validated on a set of anonymous samples provided by an independent reference center. Samples consisted mostly of biopsies and resection specimens and were derived from 54 patients positive for AL- κ , AL- λ , ATTR, or AA amyloid. The spectral classification of the samples was compared with immunohistochemical typing and clinical diagnosis (Table 3; details of the spectral classification for individual samples are reported in Supplementary Tables S9 and S10 at <http://ajp.amjpathol.org>).

Forty-five cases (1 AA, 28 AL, and 16 ATTR) clustered in a similar fashion as described above (details of the k-mean clustering analysis for all PTAA classified samples are reported as Supplemental Tables S11–S14, see <http://ajp.amjpathol.org>), and the PTAA spectral assignment correlated with the immunohistochemical typing (Figure 5 and Table 3). Again, the amyloid deposits from one case that was immunopositive for AA yielded two distinct PTAA spectra corresponding to groups 2 and 3, whereas amyloid in two other cases diagnosed as AA

showed PTAA spectra corresponding to group 2 (Figure 5 and Table 3).

Additionally, we identified three cases of amyloidosis that showed two distinct PTAA spectra in distinct regions of the respective histological specimen. Two of these cases could not be classified with immunohistochemical staining, as immunoreactivity was found for multiple antibodies. The third case displayed both PTAA spectra indicative of groups 1 (ATTR) and 2 (AL) and was found to contain distinct amyloid deposits immunoreactive with antibodies to AL and ATTR amyloid. Hence, this case appears to represent a bona fide instance of mixed AL and ATTR amyloidosis. In two cases diagnosed as AL, an extremely red-shifted emission was observed from the PTAA-stained amyloid deposits and those cases could not be classified according to any of the previously assigned groups, suggesting atypical pathogenesis. Finally, one case that proved nonclassifiable by immunotyping showed a PTAA spectrum correlating to group 2 (AL).

Discussion

Immunohistochemistry is often regarded as the technique of choice for typing of amyloid, yet it is fraught with intrinsic limitations. Since amyloids consist of very tightly packed protein aggregates, they are often poorly accessible to antibodies. This degrades the quality and signal-to-noise ratios of immunohistochemical stains. What is more, amyloid deposits often trap ancillary molecules including IgG and opsonins,³⁸ which can interact with the

Figure 4. Immunohistochemical and PTAA staining of tissue with multiple spectral profiles. **A:** Bright-field image of a liver sample from patient 8 stained with antibodies against AA amyloid. Positive staining is observed in brown. **B:** Fluorescence image of a liver sample from patient 8 stained with PTAA. Positive staining is observed in yellow and yellow-red. **C and D:** Pseudocolor visualizations of two distinct types of amyloid deposits after SUN: PTAA signals are represented in green (blue spectrum) or red (green spectrum). **E:** Representative spectra from the two different amyloid deposits stained with PTAA. **F:** Correlation diagram of the spectral ratios, $R_{538/Emax}$ and $R_{538/642}$, for PTAA from 10 different tissue samples from patient 8. All of the tissue samples had amyloid deposits with two distinct PTAA spectra showed in blue or green, respectively. The mean value of 10 spots from 5 to 10 individual amyloid deposits in each tissue section is represented by a symbol. Scale bar: 50 μ m.

anti-Ig antibodies and protein A that are used for detection. These phenomena can cause considerable difficulties in the interpretation of immunohistochemical results, and can even lead to misclassification of amyloid subtypes.^{13,20–23}

Thioflavins and Congo Red can reliably identify the presence of amyloid, but are chemically nondiscriminatory and therefore unsuitable for molecular subtyping. They also suffer from specific weaknesses^{39–41}: Congo Red staining leads to green birefringence in cross-polarized light but is not very sensitive and requires experience to reliably differentiate amyloids from a plethora of physiologically birefringent tissue constituents. The fluorescent dyes, thioflavin T and S, are more sensitive than Congo Red, but their emission maxima in the blue range can overlap with the autofluorescence of tissue components. For all these reasons, it would be desirable to expand the arsenal of techniques available for the diagnosis of amyloidoses.

Comparison between LCP Spectroscopy and Conventional Techniques

LCPs bind preferentially to protein aggregates with repetitive cross β -sheet structures.^{24–28,32} In the present study, all amyloid deposits that were identified by Congo Red stains or immunohistochemistry were also recognized by LCPs, yet the LCP fluorescence was much easier to interpret than Congo Red birefringence. Similar observations were also made when studying prion

strains, where LCP fluorescence was used to visualize nonconophilic protein aggregates.²⁷ Of all tested LCPs, p-FTAA showed a particularly strong fluorescence on binding to amyloid deposits. As a further practical advantage, the p-FTAA staining is performed in PBS, allowing costaining with antibodies and p-FTAA.

As with all generic amyloid ligands, the tinctorial selectivity of LCPs for amyloid is not absolute. Ordered macromolecular aggregates with steric properties resembling those of amyloid may interact with LCPs as well. What sets LCPs apart from Congo Red and thioflavins, however, is their capacity to undergo characteristic spectral emission shifts when associated with amyloids. This property allows for separating amyloid-associated signals from autofluorescent tissue constituents, thus preventing the detection of false positives.

Changes of the relative orientation of thiophene rings within the LCP polymer backbones, and in the degree of stacking between individual polymer chains, affect the intensity of emitted light at characteristic wavelengths.^{35–37} Since such changes can occur on binding to aggregated proteins, the spectral signatures of LCPs can be used as surrogates for the molecular structure of the respective protein aggregates. These events can be visualized by fluorescence microscopy and, more precisely, by spectral analysis.^{24–28,32} Indeed, we found that various chemically defined amyloid subtypes yielded distinct spectroscopic PTAA signatures, a fact that can be practically used for typing amyloidoses and even heterogeneous amyloid deposits in individual samples.

Table 3. Clinical Description and Tingibility of the Validation Set

Patient	Organ	Diagnosis	Age/Sex	IHC*	PTAA group [‡]
A1	Ureter	AL-amyloidosis (κ)	53/F	AL	2
A2	Rectum	AL-amyloidosis (κ)	79/M	AL	2
A3	Kidney	AL-amyloidosis (κ)	48/F	AL	2
A4	Colon	ATTR-amyloidosis	72/F	ATTR	1
A5	Liver	AL-amyloidosis (κ)	45/F	AL	2
A6	Iliac crest	AL-amyloidosis (κ)	69/M	AL	2
A7	Heart	ATTR-amyloidosis	77/M	ATTR	1
A8	Colon	ATTR-amyloidosis	71/M	ATTR	1
A9	Heart	ATTR-amyloidosis	74/M	ATTR	1
A10	Iliac crest	AL-amyloidosis (κ)	77/M	AL	2
A11	Heart	ATTR-amyloidosis	77/M	ATTR	1
A12	Heart	ATTR-amyloidosis	71/F	ATTR	1
A13	Heart	ATTR-amyloidosis	74/M	ATTR	1
A14	Heart	ATTR-amyloidosis	77/M	ATTR	1
A15	Tongue	AL-amyloidosis (κ)	58/M	AL	2
A16	Liver	AL-amyloidosis (λ)	59/M	AL	2
A17	Heart	ATTR-amyloidosis	78/M	ATTR	1
A18	Skin	AL-amyloidosis (λ)	50/F	AL	2
A19	Thyroid gland	AL-amyloidosis (λ)	47/F	AL	2
A20	Tendon	ATTR-amyloidosis	75/M	ATTR	1
A21	Heart	ATTR-amyloidosis	65/M	ATTR	1
A22	Rectum	AL-amyloidosis (λ)	54/M	AL	2
A23	Colon	AL-amyloidosis (λ)	65/F	AL	2
A24	Lung	AL-amyloidosis (λ)	77/M	AL	2
A25	Stomach	AL-amyloidosis (λ)	73/F	AL	2
A26	Heart	AL-amyloidosis (λ)	68/F	AL	2
A27	Heart	AL-amyloidosis (λ)	57/M	AL	2
A28	Heart	AL-amyloidosis (κ)	58/M	AL	2
A29	Kidney	AL-amyloidosis (λ)	74/M	AL	2
A30	Large intestine	ATTR-amyloidosis	42/M	ATTR	1
A31	Stomach/duodenum	AL-amyloidosis (λ)	55/M	AL	2
A32	Lung	AL-amyloidosis (λ)	57/F	AL	2
A33	Colon/A. temporalis	ATTR-amyloidosis	81/M	ATTR	1
A34	Liver	AL-amyloidosis (λ)	65/F	AL	2
A35	Heart	AL-amyloidosis (λ)	67/F	AL	2
A36	Stomach	AL-amyloidosis (λ)	79/M	AL	2
A37	Colon	ATTR-amyloidosis	55/M	ATTR	1
A38	Colon	AL-amyloidosis (κ)	78/M	AL	2
A39	Stomach	AL-amyloidosis (κ)	69/M	AL	2
A40	Liver	AL-amyloidosis (λ)	75/F	AL	2
A41	Rectum	ATTR-amyloidosis	86/M	ATTR	1
A42	Heart	ATTR-amyloidosis	76/M	ATTR	1
A43	Heart	ATTR-amyloidosis	70/M	ATTR	1
A44	Heart	AL amyloidosis (λ)	69/M	AL	2
A45	Duodenum	AA amyloidosis	64/M	AA	3
A46	Colon	AA amyloidosis	56/M	AA	2
A47	Stomach	AA amyloidosis	86/F	AA	2
A48	Colon	AA amyloidosis	68/M	AA	2, 3
A49	Heart	Unclassifiable case ^c	42/M	AL	2
A50	Lymph node	Unclassifiable case ^c	77/M	m. a. [†]	1, 2
A51	Skin	Unclassifiable case ^c	50/M	m. a. [†]	2, 3
A52	Heart	Mixed type of AL-λ and ATTR	73/M	ATTR and AL	1, 2
A53	Iliac crest/heart	AL-Amyloidosis (kappa)	67/F	AL	N. C.
A54	Kidney	AL-Amyloidosis (lambda)	56/F	AL	N. C.

*Amyloid subtype according to immunohistochemistry (IHC).

[†]Amyloid deposits stained for multiple antibodies (m. a.).

[‡]The numbers 1, 2, or 3 refer to the spectral classification of PTAA tingibility.

N.C. = Not classifiable; M, male; F, female.

Why does PTAA adopt different geometries on binding to specific amyloid types? Theoretical considerations⁴² and extrapolations from experiments with prion strains⁴³ strongly suggest that PTAA conformations report directly on the variability of the supramolecular structures of the cognate protein aggregates. Accordingly, LCP histochemistry represents a powerful and relatively simple method for probing the structure of complex aggregates.

Alternatively, one might speculate that the phenomena visualized by PTAA staining depend on ancillary molecules associated with amyloid deposits. Many proteins and nonprotein components, such as serum amyloid P component, heparan sulfate proteoglycan, and complement constituents, can decorate amyloid deposits.⁴⁴ The emission spectra of PTAA may be conceivably influenced by the relative contribution of these ancillary components

Figure 5. Validation of PTAA emission for amyloid typing. Correlation diagram of the spectral ratios, $R_{538/Emax}$ and $R_{538/642}$, for PTAA from 54 additional cases obtained from a second laboratory. The average value of eight spots from 5 to 10 individual amyloid deposits in each tissue section is represented as a square. The samples are visualized according to immunotyping: AA (blue squares), AL (green squares), and ATTR (red squares) amyloidoses. The mean value and the SD for each group are shown as circles with error bars. Two samples that were immunotyped as AL but that could not be classified according to PTAA emission are shown with a black box. For data for individual samples, see Supplemental Tables S9 and S10 at <http://ajp.amjpatbol.org>.

to the PTAA-stained amyloid deposits. However, distinct PTAA spectra are recorded from amyloid fibrils of different morphologies generated in recombinant, chemically defined systems lacking all ancillary constituents.²⁷ The latter observation strongly argues against the hypothesis that ancillary components specify PTAA spectra.

Spectral Classification of Systemic Amyloidoses

In total, 108 tissue samples from 96 cases were analyzed, and a perfect match between the PTAA spectral classification and the immunohistochemical typing was found for 86 (89.6%) of these cases. These data position LCP spectroscopy as a useful methodology for amyloid subtyping. Optimal typing may be reached by combining LCP spectroscopy with immunohistochemistry, since antibodies identify specific chemical entities whereas LCP spectroscopy yields information on the conformational state of protein aggregates.

The group of amyloid deposits classified as AL displayed a considerable degree of spectral heterogeneity. Furthermore, two cases (A52 and A53) showed a reddish PTAA emission profile that could not be assigned to a distinct PTAA group. The protein underlying AL amyloid is a monoclonal immunoglobulin light chain whose composition depends on the stochastic rearrangement of the V and J genomic regions during B-cell maturation. Consequently, the primary sequence of monoclonal light chains varies radically between affected individuals.^{1,45} This sequence variability is likely to translate into structural variability of AL deposits, and it was speculated that it may contribute to defining the target tissue of AL amyloid deposition.^{1,45} The spectral emission heterogeneity of PTAA-stained AL deposits is likely to be reflecting such variability. Despite their heterogeneity, the spectral characteristics from the AL-deposits were sufficiently distant from those of AA and ATTR amyloids to allow for reliable differentiation. It is tantalizing to speculate that the spe-

cific structural features of individual AL amyloid deposits, as revealed by specific PTAA emission spectra, may correlate with personalized clinical parameters.

In rare instances, more than one subtype of amyloid can coexist in the same individual. This phenomenon may be due, at least in part, to “cross-seeding” with oligomers of one protein providing a template for the aggregation of a second protein.^{46,47} We found LCP spectral assignment to be particularly useful in such instances. For example, case A52 was immunohistochemically classified as a mixed type of AL- λ and ATTR, and showed two distinct PTAA spectra corresponding to these two amyloid subtypes. Therefore, the clinical and immunohistochemical diagnosis of combined amyloidosis was verified by LCP spectroscopy. Furthermore, we found multiple PTAA spectra in two cases that eluded unambiguous immunohistochemical typing because multiple antibodies tested positive, again providing strong independent evidence that more than one subtype of amyloid was present. Confirmative LCP staining of such cases may be highly relevant to clinical management, as difficulties in the interpretation of immunohistochemistry results can lead to misclassification of amyloid subtypes.^{13,20–23}

Discrepancies between Immunotyping and PTAA Spectral Assignment

Four cases that were immunotyped as AA amyloid displayed discrepant results with the two techniques. Two of these cases showed, in distinct areas, PTAA spectra corresponding to both groups 2 and 3, whereas immunotyping revealed only AA amyloid. This result may point to limitations in the specificity of LCP spectroscopy, or alternatively it may indicate a specific supramolecular conformation of the amyloid that went undetected by immunotyping. In two additional cases, immunotyping revealed AA amyloid, yet were assigned to group 2 after PTAA spectral analysis. Maybe the atypical PTAA spectral signatures of these two cases reveal specific supramolecular conformations of AA amyloid. This is the case for prion strains, where distinct quaternary structures⁴⁸ of chemically equivalent PrP^{Sc} aggregates gave rise to distinct clinical phenotypes and, in parallel, displayed unique PTAA spectral signatures.²⁷ Analogously, the LCP emission profiles have been used for differentiation of multiple heterogeneous types of A β deposits in the brain of a transgenic mouse model.²⁸ Serum AA protein can be cleaved at multiple sites, and AA amyloid contains a diversity of truncated versions of the protein.^{14,49} In this context it will be of interest to analyze the prevalence of the various truncated forms of SAA in cases with atypical PTAA spectra.

The findings described above verify the use of LCPs as selective amyloid ligands, which provides a distinct spectroscopic read out of the conformational state of amyloid deposits. This property is not only important for verification of immunohistochemical clinical diagnosis but may also facilitate the study of amyloid origin, evolution and maturation. The latter studies are particularly

topical, since it is emerging that many misfolded proteins can act similarly to prions or “prionoids.”^{4,50–56} Furthermore, since LCPs appear to discriminate between conformational variants of protein aggregates, they may be of use in clarifying the pathogenetic importance of amyloid cross-seeding.⁵⁷ Finally, the conformation of amyloid deposits might be determined by both seed morphology and host factors.⁵³ LCP spectroscopy may provide a powerful tool to dissect this issue.

Limitations of LCP Spectroscopy

In the present study and in previous investigations of prion strains²⁷ we found that tissue fixation parameters can influence three crucial aspects of LCP histochemistry: the strength of amyloid-specific fluorescent signals, the structure-specific modulation of the emission spectra, and the amount of interfering formaldehyde-related short-wavelength autofluorescence. We found PTAA staining to be generally weaker on tissues that had been extensively exposed to fixatives, most probably as a direct consequence of excessive cross-linking of proteins with other constituents, or chemical modification of positively charged groups necessary for a strong interaction between PTAA and the amyloid deposits. This problem is by no means unique to LCPs: excessive fixation reduces dramatically the sensitivity of Congo Red stains⁵⁸ and can completely abrogate thioflavin fluorescence. Antigen retrieval techniques, which are extensively used to enhance immunohistochemical signals on formaldehyde-fixed tissues, may prove useful for enhancing LCP stainings as well. Optimized preanalytical protocols for recovery, storage, and handling of samples will be essential to translate LCP spectroscopy into a clinically useful tool.

Formaldehyde-induced autofluorescence did not appear to represent an insurmountable problem. In the present study, we observed only one TMA spot with disturbingly intense blue autofluorescence. The case in question was also negative for Congo Red staining and was excluded from the analysis for the latter reason. The spectral signature of PTAA, and therefore the molecular subtyping, is more easily affected by excessive fixation than its overall fluorescence—perhaps because cross-linking fixatives distort the structure of protein aggregates, or renders them impermeable to LCPs. In a previous study of prion deposits associated with distinct prion strains, very mildly fixed cryosections was determined to yield the most informative fluorescence spectra.²⁷ Since the availability of frozen material is often limited in routine clinical practice, the present study was performed on tissue fixed in 4% paraformaldehyde despite the caveats described above. Even under these fixation conditions, LCPs spectroscopy reliably distinguished between the three most common types of systemic amyloidoses. The highest signal-to-noise ratio and the optimal exploitation of the spectral features of LCPs, however, will only be achieved under controlled fixation conditions with specific non-cross-linking fixatives.

In conclusion, we have discovered that conformation-sensitive amyloid-binding LCPs can be used not only for

the sensitive visualization of amyloid but also for the differentiation of amyloid subtypes in tissue. The subtype-specific spectral fingerprints stem from the photophysical properties of LCPs, and cannot be obtained from any of the commonly used amyloid ligands. We foresee that LCPs, in combination with biochemical techniques and immunohistochemistry, will improve the precision of diagnoses of aggregation proteopathies, and will facilitate the study of amyloid origin and pathogenesis in humans and in animal models.

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