Chromogenic methods in coagulation diagnostics

S. Rosén
Rossix, Mölndal, Sweden

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Summary
Chromogenic peptide substrates were developed more than 30 years ago. Although the use of chromogenic substrate methods in coagulation and fibrinolysis diagnostics was not as rapidly implemented as initially believed, they are now well established for several analytes such as antithrombin, FVIII, protein C, plasminogen, plasmin inhibitor, heparins, and pentasaccharides. The advent of direct thrombin and factor Xa inhibitors has stimulated the development of new, specific chromogenic methods and these may find their way into routine use if these new drug candidates will prove to be valid replacements for coumarin derivatives. A large number of chromogenic research methods for other analytes were developed, too. The current interest in global chromogenic methods for thrombin generation and the protein C pathway may turn out as clinically important and thus enter into routine use.

Background
History
The use of a chromogenic substrate for determination of serine protease activity dates as far back as 1961 when the first compound containing a chromophore, benzoyl-arginyl-para-nitroaniline (Bz-Arg-pNA, denoted BAPNA) for determination of trypsin was published (28). This made it very simple to measure trypsin activity photometrically. However, neither the sensitivity nor the specificity was sufficient to determine coagulation enzymes such as thrombin and more than a decade elapsed until chromogenic substrates were beginning to develop as a real alternative to registration of the clotting time for determination of components in coagulation and fibrinolysis.

It all started as a thrombin inhibitor project at the former company AB Bofors Nobel-Pharma in the late 1960’s in collaboration with Blombäck’s group at the Karolinska Institute in Stockholm. From the determination of the primary structure of Fibrinopeptide A from several species, three amino acids were found to be invariant and several oligopeptide derivatives were then synthesized for structure-function studies. The small peptide Bz-Phe-Val-Arg-OMe, containing the three invariant amino acid residues, proved to have a very high affinity for thrombin and had anticoagulant properties (11); however, it was easily hydrolyzed in vivo and thus not suitable as an anticoagulant drug. Then, realizing the high affinity for thrombin and using BAPNA as a model, the peptide derivative Bz-Phe-Val-Arg-pNA (S-2160) was synthesized (87) and, with a several thousand-fold higher efficiency as compared to BAPNA as a thrombin substrate, S-2160 represented the first chromogenic peptide substrate with a sensitivity high enough for use in the haemostasis area.

Further development
After the introduction of S-2160, an intense development followed which resulted in a large number of chromogenic peptide substrates with a high selectivity and sensitivity [for a detailed presentation see ref. (98)]. Indeed, several of these substrates are more effective than the natural substrate for an enzyme, determined as the quotient between the catalytic rate and the affinity constant (kcat/Km).

One example here is the thrombin substrate H-D-Phe-Pip-Arg-pNA (S-2238), where kcat/Km is about 100-fold higher than for fibrinogen. A high selectivity is of-
ten reached by mimicking the peptide sequence preceding the cleavage site for an enzyme (Fig. 1). This is nicely illustrated by Bz-Ile-Glu-Gly-Arg-pNA (S-2222) which contains exactly the same sequence of amino acid residues which precedes both FXa cleavage sites in prothrombin (3). Interestingly, though, an even better performance both regarding sensitivity and affinity was obtained with the later developed FXa substrate Z-D-Arg-Gly-Arg-pNA (S-2765), in spite of the fact that it even has a charge difference as compared to the native tetrapeptide sequence preceding the cleavage site in prothrombin.

Other successful variants of chromogenic substrates were also developed which had a high sensitivity and which were also easily soluble in water solution, not always a trivial matter with the first generation of substrates. Examples are

- Tos-Gly-Pro-Arg-pNA (Chromozym TH) and

In some substrates, this was achieved with substitutions for natural amino acid residues and/or substituting pNA with other chromophores such as 2-methoxy-4-nitroaniline (MNA) and 5-amino-2-nitrobenzoic acid (ANBA) (48). Illustrative examples here are H-D-CHG-Abu-Arg-pNA (CBS 34.47)*, H-D-Phe-Pro-Arg-ANBA (BCP 100)* and pyro-Glu-Pro-Arg-MNA (BCP 300)*.

Another, completely different, chromophore with a very high molar extinction at 624 nm is the blue dye 7-amino-diethylamino-8-methylphenoxazine (ADMP) has also been introduced into chromogenic peptide substrates but these are not yet in routine use (85). One example where the sensitivity of chromogenic substrates appear too low is towards FVIIa. Here, fluorogenic substrates containing the fluorogen 6-amino-1-naphthalene-sulfonamide may be sufficiently sensitive, at least in the presence of tissue factor (15). Similarly, tissue factor also increases the sensitivity of a fairly recently developed chromogenic FVIIa substrate (CH₃SO₂-D-CHA-Abu-Arg-pNA (Pefachrome® FVIIa)*.

Common for most chromogenic substrates is that they have a relatively high selectivity. This may appear somewhat surprising since they after all only carry limited structural information and they are all intended for cleavage by serine proteases which are related to each other. However, it illustrates nicely that although all the target coagulation and fibrinolysis enzymes belong to the trypsin family, they have a much higher specificity than trypsin and thus a much higher requirement on the substrate structure.

Recently, a new class of substrates has been developed by linking small chromogenic peptide substrates to polyethylene glycol derivatives (44). These so called macromolecular chromogenic substrates show a relatively lower sensitivity towards ε₂-macroglobulin-protease complexes as compared to free proteases. It is yet too early to state whether these new substrates will replace the currently used to any significant extent.

**Assay design**

As mentioned, chromogenic substrates mostly show a high selectivity but they are not absolutely specific for any enzyme. Thus, it is highly important that the design of an assay will ensure a desired specificity. Interferences can often be eliminated by using a high plasma sample dilution, a feature which is made possible by the high sensitivity of most chromogenic substrates. A high sample dilution will also decrease the risk of fibrin clot formation but in some cases a fibrin polymerization inhibitor such as Gly-Pro-Arg-Pro (so-called Doolittle peptide) is included in one reagent (53). Also, by careful optimization of pH and ionic strength the specificity may be enhanced further.

* The S-series of substrates, the Chromozym/Pefachrom substrates and the BCP substrates are trademarks of, respectively, Chromogenix, Pentapharm and Dade Behring. The CBS substrates are also produced by Pentapharm but are distributed by Stago.
The wavelength 405 nm is used for determination of the amount of released chromophore although its absorbance maximum is 381 nm. The reason is that at the latter wavelength, with an absorbance maximum at 316 nm, still gives a significant contribution whereas at 405 nm the molar extinction coefficient of pNA is more than 100-fold higher than for the substrate (Fig. 2).

An important topic is the use of conditions which are in line with basic enzyme kinetics such as performing readings during the initial linear phase of the reaction and using a substrate concentration well above the affinity constant Km (18). Sometimes, this is impossible due to the inherent properties of the substrate. It is then crucial to make sure that valid results are obtained over the whole intended measuring range.

For certain enzymes such as thrombin and factor Xa, care must be taken to avoid adsorption to surfaces of test tubes or corresponding devices used in automated instruments and bulking agents such as albumin, detergents or polyethylene glycol are commonly included in reagents to minimize such events (56).

Penetration of chromogenic substrate methods

With the introduction in the early and mid 1970’s of chromogenic substrate methods for determination of blood coagulation factors it was a belief by many that the clotting methods – at that time often performed manually with the help of the stop watch – would quickly disappear (83). However, this turned out not to be the case, at least not for global tests and for several specific factor assays. There are various reasons for this such as the launch of high quality automated clotting instruments for clot registration, which made a dramatic improvement of the performance of clotting methods. Furthermore, the cost of chromogenic substrates in combination with kit designs which did not always allow an optimal usage, contributed as well to their limited rate of penetration.

In this context it should be mentioned that provided chromogenic substrate kits are properly utilized, the cost may be well below the cost for a clotting assay, as has been demonstrated for e.g. a chromogenic FVIII method (17).

Another important explanation was the fact that chromogenic PT and aPTT methods, although showing a strong performance (6, 7, 23), did not gain a widespread acceptance, to some extent possibly due to the improved standardization and therewith also a better inter-laboratory agreement for PT clotting methods with the introduction of the INR concept.

However, chromogenic methods for anti-thrombin (21, 65) and heparin (91, 92) quickly gained acceptance. Table 1 presents an overview of analytes for which chromogenic methods were published. References are also provided; however, this listing only shows examples and is by no means complete. For several of these analytes, commercial kits are available.

Coagulation diagnostics

Thrombophilia and hypercoagulability

In laboratory investigations of thrombophilia patients, it is recommended that functional activity assays are performed rather than antigen assays (94). Testing is performed of some or all (94) of:

- antithrombin,
- protein C,
- protein S,
- APC resistance due to Factor V Leiden,
- Lupus anticoagulant and/or anti-phospholipid antibodies,
- FVIII,
- prothrombin,
- dysfibrinogenemia and possibly also
- homocysteine and/or MTHFR.

Antithrombin activity is determined with chromogenic methods as residual activity of thrombin or FXa. Of importance is the finding that low antithrombin activity, over-estimation may occur due to influence of heparin cofactor II activity in thrombin-based assays and this effect is more pronounced with human as compared to bovine thrombin (32, 93). In contrast, there is no influence of heparin cofactor II in FXa-based assays (8, 24, 67).

Chromogenic methods are also available for protein C, FVIII and prothrombin; for the two latter it has been shown that elevated activities are associated with an increased thrombotic risk (49, 50, 64, 69, 100). Due to their design, chromogenic FVIII methods are insensitive to preactivation of FVIII, which is of importance as regards accuracy (73).

Free (rather than total) protein S deficiency constitutes another risk factor but its real risk is not clearly established, in part probably due to that an estimation to determine protein S deficiency in the general population has only been published fairly recently (27). No chromogenic method exists for free protein S deficiency but the need for such a method may be limited due to the availability of a sensitive immuno assay method based upon binding of free protein S to its natural ligand C4bBP (36). Although being an immunoassay it seems to reflect
properly the biological functional activity of free protein S.

As regards APC resistance due to FV Leiden (Arg506Gln), clotting methods and gene testing are dominating. However, sensitive and specific chromogenic methods were also published but are not yet in routine use (62, 72). It should be noticed that by using PCR for detecting a FV abnormality, cases of so called pseudo-homozygous FV Leiden (39) will not be identified but classified as regular heterozygotes for the Arg506Gln mutation whereas the functional aPTT-based clotting test classifies it as a homozygous defect and the same classification may well be obtained also with a chromogenic method. A correct identification of such patients is of importance since they seem to carry the same risk as a homozygous FV Leiden individual.

Regarding fibrinolytic components, investigation for plasminogen deficiency as a cause of thrombophilia is not commonly performed since it is very rare, at least in the Caucasian population. Furthermore, systematically low t-PA and high PAI-1 activities have not been clearly shown to be associated with an increased risk of venous thrombosis (58, 71). However, sensitive chromogenic methods are available for these analytes. In case of determination of t-PA activity in plasma, it is of crucial importance to use immediate acidification to prevent ex vivo inactivation by PAI-1 (75). The interesting area of cancer disease and the use of tPA, t-PA and PAI-1 as prognostic markers is discussed by Menenge et al. in this issue (pp. 301-10).

Another interesting analyte is tissue factor pathway inhibitor (TFPI). This component will be dealt with elsewhere in this issue by Dr. Alban (pp. 286-92) in detail. Suffice to mention here that a complete deficiency of TFPI appears to be a very serious state (77).

Activated platelets, monocytes and microparticles display procoagulant activity to various extent and much research is currently devoted to these cellular components, especially so as regards microparticles (31). Chromogenic methods are thereby often used and the effect of exposed negatively charged phospholipids and/or tissue factor is thereby tested for their stimulation of FXa or thrombin generation (2, 4, 55, 63, 89). One difficulty is that tissue factor may be present on microparticles as an encrypted molecule and more research is needed to identify whether it will be clinically valuable to monitor this procoagulant activity in some disease states.

One direct sign of hypercoagulability is elevated levels of soluble fibrin. Persistently high levels of soluble fibrin, measured with a chromogenic method (97), were associated with a bad prognosis (14).

It was mentioned that chromogenic methods for the global assays aPTT and PT has not gained a wide acceptance. However, this may possibly be the case for the thrombin generation test, which is gaining an increasing interest. Here, a chromogenic thrombin substrate with low sensitivity and a low affinity is utilized (43) and this test can also be applied on platelet rich plasma, albeit then utilizing a fluorescent substrate (42). So far, however, the test is not easily applied on commonly available automated instruments, which may hamper its entrance into routine use. In an interesting variant of this method, the thrombin potential is determined in the absence and presence of APC (74).

This test not only has a high sensitivity for protein S deficiency but also for APC resistance phenotype not due to FV Leiden, and in initial studies the extent of this abnormality seems to correlate with thrombotic risk (22). Recently, another chromogenic method for determination of abnormalities in the protein C pathway was published, which also appear promising (76).

Finally, recent studies on FXII, high molecular weight kininogen (HMWK) and prekallikrein (35) indicate that these factors may deserve further attention in venous thrombosis.

**Therapy**

Chromogenic methods are also used for monitoring of some therapeutic substances, the dominating applications being anti-FXa methods for unfractionated and low molecular weight heparins.

In the latter case, the clinical value of monitoring may not be obvious from some studies (13, 37) but nevertheless such assays are performed to a great extent and seemingly required in at least some patient categories (41).

A recently introduced pentasaccharide is also analysed by anti-FXa assays (25) and this matter is further discussed by Dämgen von Bovern in this issue (pp. 281-5). Other examples on the use of chromogenic methods are determination of FVIII concentrates, for which purpose the chromogenic method is recommended by ISTH (5) and it is as well the European Pharmacopoeian reference method.

Although a chromogenic FIX method was published many years ago (88), it does not seem to be used for haemophilia B diagnosis. However, low levels of contaminating FIXa in FIX concentrates may be determined, either by a coupled amidolytic FXa assay (38) or with the use of a recent direct substrate for FIXa (70).

A rare cause of bleeding is deficiency of PAI-1, which as already mentioned can be detected by a chromogenic method (26).

**Bleeding**

Only a limited number of chromogenic methods are available for investigation of a bleeding diathesis. Functional plasmin inhibitor deficiency is almost exclusively determined with this approach and recently the method has been modified by including methylene blue to eliminate the influence of α2-macroglobulin (10).
low, chromogenic methods have been developed and applied in various studies (40, 47, 68, 82). The determination of hirudin with a snake venom based assay is presented by Dr. Lange et al. in this issue (pp. 293-300). Monitoring of coumarin therapy by the PT method can be difficult in lupus anticoagulant patients and the use of a chromogenic FX method has been suggested (60).

**Conclusion**

During the last 30 years, the use of chromogenic substrates has had a great impact on our evolving knowledge of haemostasis. Their role in the coagulation laboratory is well established and will probably remain. Extensive clinical evaluation of chromogenic global assays for thrombin generation and the protein C pathway will demonstrate whether these will justify a place in routine use. Currently, an increasing user convenience is achieved through the use of e.g. properly formatted, bar-coded as well as stable, ready-for-use, liquid reagents.

**References**


Correspondence to:
Dr. Steffen Rosén
Rossix, Toljängögatan 3B
431 53 Mölndal, Sweden
Tel. +46/31/70 68 69, Fax -66
E-mail: rossix@telia.com