



Hydrogels thermosensibles et mucoadhesifs : nouvelles stratégies pour prévenir et traiter les pathogènes au niveau de la muqueuse vaginale

Bénédicte Pradines

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Bénédicte PRADINES

**Hydrogels thermosensibles et mucoadhésifs :
nouvelles stratégies pour prévenir et traiter les
pathogènes au niveau de la muqueuse vaginale**

Directeur de thèse :

Co-directeur de thèse :

Philippe LOISEAU

Kawthar BOUCHEMAL

Professeur (Université Paris-Sud)

MCF (Université Paris-Sud)

Composition du jury :

Rapporteurs :

Examineurs :

Didier BETBEDER

Philippe VINCENDEAU

Madeleine DJABOUROV

Christine VAUTHIER

Professeur (Université Lille 2)

Professeur (Université Bordeaux 2)

Professeur (ESPCI Paris)

DR CNRS (Université Paris-Sud)

Hydrogels thermosensibles et mucoadhésifs :
nouvelles stratégies pour prévenir et traiter les
pathogènes au niveau de la muqueuse vaginale

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Abréviations et définitions

Abréviations et définitions

Liste des abréviations

AIDS	acquired immunodeficiency syndrome
AP	attachment protein
<i>C. albicans</i>	<i>Candida albicans</i>
CDC	Center for disease control and prevention
CP	cysteine protease
DNA	deoxyribonucleic acid
FDA	Food and Drug Administration
HIV	human immunodeficiency virus
Ig	immunoglobulin
IL	interleukine
LPG	lipophosphoglycan
MAPK	MAPKinases
MLC	minimum lethal concentration
mRNA	messenger ribonucleic acid
PFOR	pyruvate:ferredoxin oxidoreductase
RNA	ribonucleic acid
STD	sexually transmitted disease
STI	sexually transmitted infection
TLR	tool-like receptor
<i>T. vaginalis</i>	<i>Trichomonas vaginalis</i>
USA	United States of America
Vec	vaginal epithelial cells
WHO	World Health Organization

Définitions

Adnexitis: inflammation of the adnexal organs of the uterus, such as the ovaries or fallopian tubes (Mosby and O'Toole 2013)

Convulsive seizure: a sudden onset of a disease, characterized by convulsions, palpitations, and other symptoms. The term is sometimes applied to an attack of an epileptic disorder (Mosby and O'Toole 2013)

Dyspareunia: difficult or painful sexual intercourse (Dorland 2012)

Dysuria: painful or difficult urination (Dorland 2012)

Eosinophilia: an absolute or relative increase in the normal number of eosinophils in the circulating blood. Various limits are given (e.g., absolute eosinophilia if the total number exceeds 500 mm^3) and relative if greater than 3% but total less than 500 mm^3 . It may be associated with skin diseases, infestations, hay fever, asthma, angioneurotic edema, adrenocortical insufficiency, and Hodgkin disease (Mosby and O'Toole 2013)

Incidence: (in epidemiology) the number of new cases in a particular period. Incidence is often expressed as a ratio, in which the number of cases is the numerator and the population at risk is the denominator (Mosby and O'Toole 2013)

Leukopenia: a decrease in the normal number of white blood cells in the circulating blood. Various lower limits are given; e.g., leukopenia signifies less than 4000 white blood cells per cubic millimeter (Mosby and O'Toole 2013)

Neoplasia: the new and abnormal development of cells that may be benign or malignant (Mosby and O'Toole 2013)

Peripheral neuropathy: The term peripheral neuropathy encompasses a wide range of disorders in which the nerves outside of the brain and spinal cord—peripheral nerves—have been damaged. Peripheral neuropathy may also be referred to as peripheral neuritis, or if many nerves are involved, the terms polyneuropathy or polyneuritis may be used (Mosby and O'Toole 2013)

Prevalence: (in epidemiology) the number of all new and old cases of a disease or occurrences of an event during a particular period. Prevalence is expressed as a ratio in which the number of events is the numerator and the population at risk is the denominator. (Mosby and O'Toole 2013)

Pruritus: the symptom of itching, an uncomfortable sensation leading to the urge to scratch. Scratching may result in secondary infection. Some causes of pruritus are allergy, infection, jaundice, chronic renal disease, lymphoma, and skin irritation. Treatment is best directed at the cause. Symptomatic relief may be obtained with antihistamines, starch baths, topical corticosteroids, cool water, or alcohol applications. (Mosby and O'Toole 2013)

Pruritus vulvae: intense itching of the female external genitals (Dorland 2012)

Pyosalpinx: accumulation of pus in a fallopian tube (Mosby and O'Toole 2013)

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Introduction

Introduction

Selon les dernières estimations de l'OMS, on enregistre chaque année dans le monde 498,9 millions de nouveaux cas d'infections sexuellement transmissibles (IST) dont 276,4 millions sont dus au parasite *Trichomonas vaginalis* (*T. vaginalis*) (Rowley et al. 2012). Au niveau du tractus génital, la colonisation et l'irritation de la muqueuse vaginale par *T. vaginalis* favorisent la survenue de complications infectieuses comme les chlamydie ou les gonococcie. Ces infections associées peuvent conduire à des infections chroniques et avoir à terme des conséquences graves comme la stérilité, la rupture prématurée des membranes ou encore la mort prématurée du nourrisson (Cotch 1990; Cotch et al. 1997; Hardy et al. 1984; Minkoff et al. 1984; Carlier et al. 2012). De plus, ces infections vaginales sont des facteurs de risque qui favorisent les infections par le Virus d'Immunodéficience Humaine (VIH-1) (Laga, Nzila, and Goeman 1991; Fox and Fidler 2010; Nweze and Mouneke 2011).

Actuellement, la famille chimique des nitro-5 imidazoles est la seule classe de médicaments utilisée comme thérapie orale ou parentérale de la trichomonose urogénitale. Cependant, la survenue de résistances aux nitroimidazoles et leur toxicité justifient des approches alternatives. Ainsi, l'objectif de ce projet est de développer de nouvelles formulations à action locale pour la prévention et le traitement des pathogènes qui colonisent les muqueuses vaginales (*T. vaginalis* et *C. albicans*).

La voie vaginale est cependant très exigeante et malheureusement à ce jour la majorité des formulations existantes ne répondent pas aux besoins des femmes. Pour être pleinement efficace, une formulation microbicide topique doit empêcher l'interaction des pathogènes avec les cellules cibles.

Dans ce contexte, l'objectif de notre travail consiste à concevoir et à développer un système modèle permettant de traiter localement et simultanément plusieurs infections qui toucheraient la voie vaginale. Pour atteindre cet objectif nous avons développé des gels de pluronic® F127 destinés au traitement et à la prévention locale de l'infection par *T. vaginalis* ou *C. albicans*. Un système nanoparticulaire composé de poly(cyanoacrylate d'isobutyle) (PIBCA) a également été étudié pour l'encapsulation et la libération de principes actifs.

Notre travail est présenté de la manière suivante. Dans une première partie nous essaierons de faire le tour de la problématique liée aux traitements oraux ou parentéraux de la trichomonose urogénitale en donnant un bref aperçu de l'état des lieux et les perspectives en matière de recherche sur le développement de traitements des infections à *T. vaginalis*. Nous expliquerons pourquoi la voie vaginale nous a semblée être la meilleure alternative aux traitements existants. Dans ce premier chapitre, nous poserons les bases anatomo-physiologiques concernant la voie vaginale, les mécanismes mis en jeu lors de l'infection vaginale à *T. vaginalis*, ainsi que les données galéniques des formulations adaptées à cette voie.

La deuxième partie du mémoire, essentiellement expérimentale, est consacrée à décrire la formulation mucoadhésive et thermogélifiante que nous avons conçue pour l'administration locale du métronidazole, de l'albendazole et du clotrimazole. Les formulations que nous proposons sont composées d'une molécule active incluse dans des cyclodextrines qui seront elle-même incluses dans des nanoparticules de PIBCA qui seront ensuite dispersées dans un hydrogel thermogélifiant à base de pluronic® F127 et de chitosane.

Tout d'abord, nous nous sommes intéressés à augmenter la solubilité apparente de ces molécules en milieux aqueux en faisant appel à des cyclodextrines (CDs). Par la suite, la possibilité d'encapsuler ces molécules dans un système nanoparticulaire mucoadhésif a été

explorée. Avant l'encapsulation, une étude physico-chimique et rhéologique a été menée pour déterminer l'impact de l'inclusion des NPs vides dans un hydrogel de Pluronic[®] F127. A chaque étape de la formulation la toxicité a été évaluée sur différentes lignées cellulaires et de la muqueuse vaginale porcine. Il s'agit de la lignée HeLa provenant d'un adénocarcinome du col de l'utérus humain et des lignées Caco-2/TC7 et HT-29/MTX provenant d'un adénocarcinome colique humain. Ces études de toxicité ont été décisives dans nos choix de formulation et nous ont menée à la formulation d'un gel plus simple composé de pluronic[®] F127, de chitosan et de métronidazole qui est une molécule active contre *T. vaginalis*. Des études de libérations ont été effectuées en utilisant des cellules de Franz puis l'activité anti *T. vaginalis* a été testée *in vitro* sur des cultures en milieu liquide et sur des co-cultures HeLa/*T. vaginalis*.

Une discussion générale conclut le mémoire en permettant de mettre en perspectives les résultats obtenus, puis de dégager les avantages et les inconvénients de notre stratégie.

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TRAVAUX ANTERIEURS

Strategies for the prevention and the treatment of
urogenital trichomoniasis

Strategies for the prevention and the treatment of urogenital trichomoniasis

1. Introduction

Trichomoniasis is an extremely common cosmopolitan genitourinary sexually transmitted infection (STI) in humans caused by the parasite *Trichomonas vaginalis* (*T. vaginalis*). This disease has important medical, social, and economical implications. Trichomoniasis is reported to be a major cause of pathology in obstetrics and gynecology. This disease usually remains localized to the lower part of urogenital area but can have important consequences like upper reproductive tract post-surgical infections, reversible infertility, premature rupture of the placental membranes, premature labor, low-birth-weight infants, neonatal morbidity and mortality (Cotch 1990; Cotch et al. 1997; Hardy et al. 1984; Minkoff et al. 1984; Carlier et al. 2012).

Moreover, *T. vaginalis* has been described in participating in HIV (human immunodeficiency virus) transmission because of the lesions provoked by the parasite (Fox and Fidler 2010; M Laga, Nzila, and Goeman 1991).

Currently, the nitroimidazole chemical family is the only class of drugs useful as oral or parenteral therapy of trichomoniasis. However, nitroimidazole-resistance and toxicity justify alternative approaches. This review gathers present strategies both at experimental and clinical levels for the urogenital trichomoniasis treatment.

2. *Trichomonas vaginalis*

2.1. Biology of *Trichomonas vaginalis*

Trichomonas vaginalis is the most widely studied parasite of all trichomonads. *T. vaginalis* is a flagellated protozoa from the order of Trichomonadida, the class of Zoomastigophorea Parabasalia the Subphylum of Mastigophora, the Phylum of Sarcomastigophora Metamonada and the Kingdom of Protista (Donne 1836). The size and the shape of the parasite can be highly variable depending of the growth condition, but typically appears with the average length and width being 9-23 and 7 μm (average 13 μm) respectively (Benchimol 2004). *T. vaginalis* is a parasitic protozoan, typically pyriform but physicochemical conditions do alter the appearance of the parasite. In axenic culture, the shape of the parasite tends to be uniform, pear shaped or oval, but the parasite takes on a more amoeboid appearance after attachment to vaginal epithelial cells (Gould et al. 2013).

T. vaginalis is a flagellated protozoan possessing five flagella. Four flagella of about 7 to 18 μm are located at its anterior portion and give its characteristic twisting and wriggling movement. The fifth flagellum is incorporated within the undulating membrane which length is equivalent to half those of the cell and supported by a slender non contractile costa. An axial axostyle starting centrosome is extended by a small posterior tip end (Figure 1).

The life cycle of *T. vaginalis* is simple in that the trophozoite is transmitted through coitus and no cyst form is known. *T. vaginalis* is extracellular to genitourinary tract epithelium with a primarily anaerobic lifestyle and its multiplies by longitudinal binary fission.

T. vaginalis has a large nucleus characteristic of eukaryotic cell as well as a highly developed Golgi. *T. vaginalis* is a member of the parabasilid lineage of microaerophilic

eukaryotes, without mitochondria but it contains a hydrogenosome as an alternative provider of energy. Hydrogenosomes are double-membrane-bound organelles involved in catabolic process, including glycolysis and concentrated primarily around the axostyle and the costa (Morada et al. 2011; Wright et al. 2010). It produces ATP and hydrogen via the decarboxylation of pyruvate using the key enzyme pyruvate:ferredoxin oxidoreductase (PFOR) (Kulda 1999).

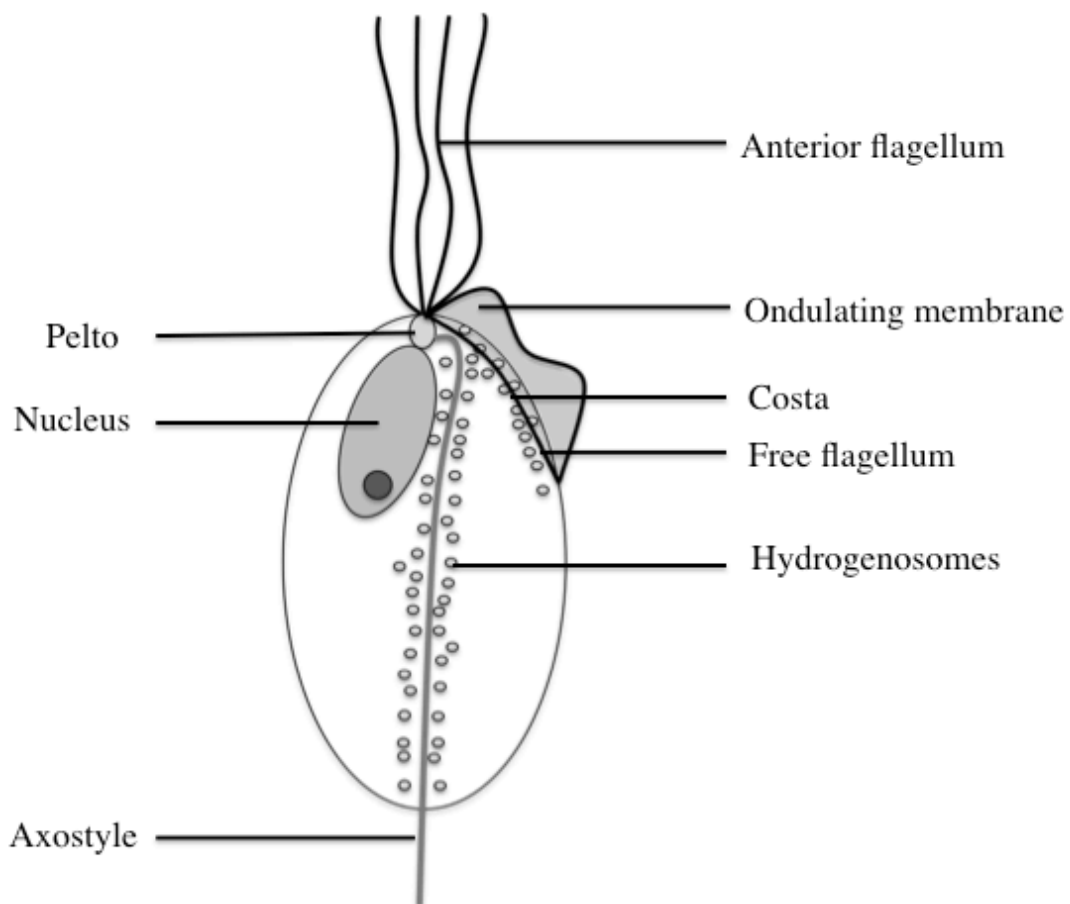


Figure 1: Schematic drawing of *Trichomonas vaginalis*

2.2. Epidemiology and transmission

Humans are the only natural hosts of *T. vaginalis*. Trichomoniasis is an extremely common infection worldwide. The World Health Organization (WHO) estimates that the total number of new cases of curable STIs in 2008 in adults between the ages of 15 and 49 was about 498.9 million with 276.4 million cases resulting from *T. vaginalis*. In addition, it was estimated that 187.0 million adults were infected with *T. vaginalis*. The 2008 estimate of the number of new cases of infection with *T. vaginalis* is 11.2% higher than for 2005 (276.4 million versus 248.5 million) (Rowley et al. 2012).

The prevalence* and the incidence* of vaginal *T. vaginalis* infection vary as a function of the geographic location. These values were significantly higher in Africa and America than the other parts of the world (Table 1). Moreover, a Danish study using *T. vaginalis* culture data collected between 1967 and 1997, showed that the prevalence of *T. vaginalis* in women may be declining in industrialized nations (Dragsted, Farholt, and Lind 2001). Similarly various Iranian studies have determined the prevalence of *T. vaginalis* infection to be between 2 to 8%, but based on cultural and social status, this rate can be higher than 30% (Matini et al. 2012).

* Cf definitions

Table 1: Incidence and Prevalence of *T. vaginalis* infection in woman between the ages of 15 and 49 in different World regions in 2008 (data from WHO(Rowley et al. 2012))

Regions	Incidence		Prevalence	
	Per 1000	Millions	%	Millions
Africa	146	28.1	20.2	38.9
America	177.7	42.5	22.0	52.7
South-East Asia	40.3	18.5	5.6	25.7
Europe	51.7	11.6	5.8	13.0
Eastern Mediterranean region	64.0	9.7	8.0	12.0
Western Pacific region	45.6	21.9	5.7	27.2

Trichomoniasis is a venereal disease affecting the two sexes. The importance of sexual contact in the transmission of *T. vaginalis* is well established (Swygard et al. 2004). Although *T. vaginalis* can survive in body fluids and on moist surface, there is not existing well-documented cases of non-venereal transmission (Lewis 2010). *T. vaginalis* is known to be congenitally transmitted from infected pregnant women to their fetuses (Carlier et al. 2012). In fact, some rare case of vaginal, urinary tract, nasal and respiratory infection with *T. vaginalis* have been reported in newborns infected with this parasite (Schwandt, Williams, and Beigi 2014; Temesvári and Kerekes 2004). Unlike other non-viral STI, trichomoniasis does not primarily reach young women (ages 15-25) but women during the reproductive years, the highest rates of *T. vaginalis* infection being found in women between the ages of 35 and 40 (Bowden et al. 1999; Cudmore et al. 2004). Predisposing factors include African-American race, older age, active drug use, non-use of condoms or oral contraceptives, trading sex, multiple sex partners, smoking, single marital status and low socioeconomic class (Lewis 2010; Nanda et al. 2006). HIV infection has not been associated with higher rates of vaginal trichomoniasis in the USA (Nanda et al. 2006), however a study conducted in four cities in

Africa showed that the prevalence of vaginal trichomoniasis was significantly higher in the cities with higher rates of HIV infection (Buve et al. 2001). The average duration of infection depends the health care seeking behavior of the population, and access to health care.

2.3. *Trichomonas vaginalis* infection

2.3.1. Clinical presentation

T. vaginalis is usually isolated from the vagina but the urethra and Skene's gland are also commonly infected (Wølner-Hanssen P et al. 1989).

The infection, once established, may persist for a long period in women. Asymptomatic *T. vaginalis* infections are well documented, up to 25 to 50% of infected women do not show clinical sign, however one-third of these will develop symptoms within 6 months. The symptoms may be cyclic and often get worse during menstruation (Petrin et al. 1998) For 50% of infected women the incubation period is 4 to 28 days (Catterall 1972).

Symptoms in women include purulent vaginal discharge (thin, greenish-yellow, frothy and mucopurulent), pruritus* in or near the vagina, dyspareunia*, vaginal odor (foul or strong smell), dysuria*. The classical discharge from the vagina is greenish-yellow, frothy, itchy and malodorous, however in clinical practice the discharge is often grey and non-itchy (Lewis 2010). In women with culture-proven *T. vaginalis* infection only 11-17% present abnormal discharge, odor, pruritus, dysuria or vaginal burning (Landers et al. 2004). Small punctate

* Cf definitions

hemorrhagic spots may be found on the vaginal and cervical mucosa, and a “strawberry cervix” is observed in only 2% of women (Fouts and Kraus 1980).

Trichomoniasis is characterized by a rise in the vaginal pH from approximately 4 to as high 7 (Anorlu et al. 2001). This rise of pH is induced by a decrease or elimination of endogenous *Lactobacillus* species and creates a better environment for the growth of the parasite (Goode, Grauer, and Gums 1994). Moreover, some study show that some factors participate to pathogenicity such as cell detaching factor, are inactivated at pH of less than 5. It was also proved that the activity of cell detaching factor was inhibited by estrogen (Garber, Lemchuk-Favel, and Rousseau 1991). The fact that the trichomoniasis symptoms are worse during menstruation can be explained by the roles played by pH and hormones. Furthermore menstrual blood creates a rich medium with high rate of iron at higher pH than as normal in the vagina, that promotes *T. vaginalis* reproduction and attachment to the vaginal epithelium and contribute to the exacerbation of symptoms at this time (Harp and Chowdhury 2011; Petrin et al. 1998).

2.3.2. *Diagnosis*

The data suggest that diagnosis cannot be based simply on the symptoms; laboratory investigation is necessary to determine precisely trichomoniasis. Microscopic observation first described in 1836 (Donne 1836) and measurement of the vaginal pH provide additional information essential to a reliable diagnostic. Currently, the “gold standard” for the diagnosis of trichomoniasis is culture (95% sensitivity) but it requires the use of a specialized medium, such as Diamond’s medium, with a prolonged incubation of up to several days (Kingston, Bansal, and Carlin 2003). *T. vaginalis* Polymerase chain reaction (PCR)-based test provides a

good alternative to culture and can detect very few trichomonads in a sample explaining a sensitivity greater than 65% and the specificity greater than 97% (Hobbs et al. 2006).

The FDA has approved two other tests for *T. vaginalis* diagnosis in woman. The OSOM[®] *T. vaginalis* rapid antigen test (Genzyme Diagnosis, Cambridge, MA, USA) is an immunochromatographic capillary flow dipstick that detect *T. vaginalis* membrane protein within 10 minutes. The Affirm[™] VPIII test (Becton Dickinson, Sparks, Maryland, USA) is a nucleic acid (RNA) amplification that detect *T. vaginalis*, *Gardnerella vaginalis* and *Candida albicans* in 30-60 minutes (DeMeo et al. 1996; Huppert et al. 2005). These test out-perform wet preparation and demonstrate sensitivity greater than 90% and specificity greater than 99% (Harp and Chowdhury 2011).

Recently, the commercially availability of nucleic acid amplification tests (NAATs) has been validated in asymptomatic and symptomatic women and are a highly sensitive test that can be used on multiple specimen types including urine, vaginal specimens, endocervical specimens and endocervical ThinPrep specimens. (J. R. Schwebke et al. 2011).

2.3.3. Complications

Even if trichomoniasis usually remains localized in the lower part of urogenital area, it can occasionally provoke adnexitis* or pyosalpinx* (Sebek 1972) and may potentially have serious sequel in women and especially during pregnancy.

T. vaginalis infections in pregnancy have been shown to be associated with low birth weight, preterm delivery, preterm delivery of a low birth weight infant, premature rupture of

* Cf definitions

the placenta membranes, stillbirth and neonatal birth (Cotch 1990; Cotch et al. 1997; Hardy et al. 1984; Minkoff et al. 1984).

Trichomoniasis is associated with an increased risk of acquiring HIV and co-infection with other STIs (Marie Laga et al. 1993). A Rwandan study showed a significant difference between the prevalence of *T. vaginalis* infection in pregnant women infected with HIV or not (20.2% and 10.9 % respectively) (Leroy et al. 1995). A study comparing *T. vaginalis* in African-American communities of the USA to other treatable STIs demonstrate that *T. vaginalis* consequently contribute to HIV acquisition and exceed the relative contribution of other STIs (Sorvillo et al. 2001). Similarly, it has been demonstrated that metronidazole use for trichomoniasis decrease significantly HIV free-cells (RNA) (Wang et al. 2001). Moreover, complications of upper genital tract infection in HIV infected women increase significantly with *T. vaginalis* and can decrease if *T. vaginalis* infection is controlled (Fox and Fidler 2010; Moodley et al. 2002).

A longitudinal study evaluating *T. vaginalis* and human papillomavirus interactions showed an association between *T. vaginalis* infection and cervical intraepithelial neoplasia* (Gram et al. 1992).

Finally, several studies have shown that *T. vaginalis* infection present a risk factor for tubal infertility (Grodstein, Goldman, and Cramer 1993; Grys 1973; Sherman, Daling, and Weiss 1987; Sherman et al. 1988; Soper 2004).

* Cf definitions

2.3.4. Adhesion

T. vaginalis infects the squamous epithelium of the vaginal tract and its adherence to vaginal epithelial cells (Vec) is the initial step leading to infection in woman (Moya, Su, and Honek 2009). Attachment to cells is time-, temperature- and pH-dependent. After cytoadherence, *T. vaginalis* changes its structure to get an amoeboid form which allows it increasing contact surface with Vec and forms cytoplasmic projections that interdigitates with target cells (Rossana Arroyo et al. 1993; Caillouette et al. 1997).

Five *T. vaginalis* attachment proteins (AP) able to mediate the parasite attachment to Vec have been identified: AP23, AP33, AP51, AP65 and AP120. All are surface glycoproteins that have high identity to hydrogenosome metabolic enzymes and are induced by iron (Engbring, O'Brien, and Alderete 1996; Meza-Cervantez et al. 2011; Moreno-Brito et al. 2005). AP33 is composed of two distinct domains, one in the N-terminal half of the protein, and the other within 24 residues in the C-terminal third of the protein (Engbring and Alderete 1998b, 33). AP33 has significant sequence identity to the succinyl-CoA synthetase α -subunit of several different organisms and the characteristics of the AP33 protein are similar to those of the other adhesins and emphasize a complex host–parasite relationship (Engbring and Alderete 1998a). AP65 is a prominent *T. vaginalis* adhesin (A. S. Kucknoor, Mundodi, and Alderete 2005a). The *ap65-1* gene belongs to the *AP65* multigene family encoding multiple homologous 65-kDa proteins which have protein sequences identical to those of the hydrogenosomal malic enzymes (Ong et al. 2006).

Other *T. vaginalis* surface proteins allow association with Vec including fibronectine binding protein, laminin binding protein and glycolipids such as lipophosphoglycan (LPG)

(Harp and Chowdhury 2011). The *T. vaginalis* LPG is the most abundant glycoconjugate on the parasite surface ($2-3 \times 10^6$ copies/parasites). LPG is a pure carbolipid (no peptide component) that similarly to prokaryotic glycoconjugates anchored on the parasite surface via inositol-phosphoceramides (Fichorova 2009; Singh et al. 2009).

T. vaginalis adhesion to the human vaginal epithelial cells is species specific and capable of inducing gene upregulation not only in the parasite (A. S. Kucknoor, Mundodi, and Alderete 2005b) but also in the host (Fichorova et al. 2006; A. Kucknoor, Mundodi, and Alderete 2005). Immunoinflammatory responses to *T. vaginalis* infection have been observed in woman as discussed below.

The possibility of using formulations that inhibit the parasite adhesion to the vaginal epithelium and preserve the vaginal normal pH may prove to be a substantive new approach to the treatment of urogenital trichomoniasis.

2.3.5. Cysteine proteinases (CP) and cytotoxic molecules

T. vaginalis has abundant proteinases and more than 400 distinct proteinase genes of the genome have been reported. Up to 220 correspond to the cysteine type (Carlton et al. 2007), and up to 23 spots with proteolytic activity between 23 and 110 kDa are detected using two-dimensional (2D) substrate gel electrophoresis (zymograms) (Neale and Alderete 1990). However, most of these cysteine proteases (CP) are encoded by only nine distinct CP genes (Ramón-Luig et al. 2011). Some of these genes are modulated by environmental factors such as pH (Petrin et al. 1998), polyamines (Alvarez-Sánchez et al. 2008) and iron (Cárdenas-Guerra et al. 2013; León-Sicaire, León-Félix, and Arroyo 2004; Rendón-Gandarilla et al. 2013; Torres-Romero and Arroyo 2009). These genes regulation plays decisive roles in

characteristics related to *T. vaginalis* virulence including cytotoxicity (Alvarez-Sánchez et al. 2008; Rossana Arroyo et al. 1993; Ramón-Luing et al. 2011, 39), haemolysis (Cárdenas-Guerra et al. 2013; Dailey, Te-Hung, and Alderete 1990; Fiori et al. 1993; Fiori et al. 1996), cytoadherence (R. Arroyo and Alderete 1994; Hernández et al. 2004; Mendoza-López et al. 2000; Rendón-Gandarilla et al. 2013), complement resistance (Alderete, Provenzano, and Leherker 1995), immune evasion and apoptosis in human cells (Cuervo, Fernandes, and de Jesus 2011). In addition, these enzymes are detected in whole cell extracts and secretions of the parasite (De Jesus et al. 2007). Several studies have shown that both secreted and cell-surface CPs of *T. vaginalis* contribute to the cytotoxicity to HeLa cells (human cervical carcinoma cells). Thus, a secreted fraction called CP30 was directly related with apoptotic events in epithelial cells (Sommer et al. 2005). These cytoplasmic CP have trypsin like activity and function as cell-detaching factors by degrading protein (such as laminin or fibronectin) of the extracellular matrix and facilitate the release of host cell from tissue, resulting in desquamation of the vaginal and cervical epithelia (Harp and Chowdhury 2011).

For *T. vaginalis*, lipid and iron from erythrocytes are an essential source of nutrient. *T. vaginalis* phagocytizes and lyses erythrocytes by a contact-dependent mechanism (Dailey, Te-Hung, and Alderete 1990; Lustig et al. 2013; Rendón-Maldonado et al. 1998). Haemolysis requires calcium and an acidic pH (6.5) at 37 °C. Among the molecules possibly involved in *T. vaginalis* haemolysis, pore-forming proteins and phospholipase-A-like proteins have been identified as cytolytic factors (Fiori, Rappelli, and Addis 1999; Lubick and Burgess 2004). Furthermore, CPs participate in haemolysis, potentially through a contact-dependent mechanism (Dailey, Te-Hung, and Alderete 1990).

Some CPs could be potential biomarkers for serodiagnosis of trichomonosis (Ramón-Luing et al. 2011).

2.3.6. Immunity and immune system evasion

T. vaginalis can survive and flourish in the hostile environment of the vagina. Its capacity to evade the host immune system is an important aspect of its pathogenicity. *T. vaginalis* is an extracellular parasite and its elimination will most likely be immunoglobulin (Ig)-dependent (Smith and Garber 2013). The immunoinflammatory response to *T. vaginalis* infection have been studied *in vitro* and in different animal models including murine, bovine and nonhuman primate models (Abraham et al. 1996; Cobo et al. 2009; Kulda 1990; Patton et al. 2006). *In vitro* experiments have been performed using cervical and vaginal cells (Bastida-Corcuera et al. 2005; J.-H. Chang, Park, and Kim 2006; Fichorova et al. 2006; A. S. Kucknoor, Mundodi, and Alderete 2005a; A. S. Kucknoor, Mundodi, and Alderete 2005b; A. S. Kucknoor, Mundodi, and Alderete 2007; Sommer et al. 2005) and various immune cell types (J.-H. Chang et al. 2004; Ryu et al. 2004; Shaio et al. 1995). The most physiological model for the study of *T.vaginalis* infection are human cervical and vaginal epithelial cells because they are the host cells supporting the parasite adherence, survival, and replication in the genital tract (Fichorova 2009).

In the vaginal discharge of women infected with *T. vaginalis*, a large number of polymorphonuclear leukocytes have been found (Shaio et al. 1995). Histologically, pronounced mast cell response is one of the characteristic features in the vaginal tissues during infection with *T. vaginalis* (Kobayashi et al. 1982). As with many sexually transmitted diseases, infection with *T. vaginalis* does not induce long-term immune protection (Adagu et al. 2002; Honigberg 1987). A complicated host defense system restricts *T. vaginalis* infection to the genitourinary tract and may play a role in limiting the immune response and preventing long-term immunity (Cudmore et al. 2004). The ability to cleave host immunoglobulins by parasite proteases may contribute to the immune evasion of the parasite with complex

interaction similar to the situation for other cell-cell contacts (Gomez et al. 2010).

During the process of infection, *T. vaginalis* is associated with epithelial cells. In the previous paragraph, we discussed about the receptors in the parasite-cells interactions. This active involvement of signaling pathways leads to the production of IL-8, IL-6, MCP-1 and TNF α . The signaling pathways that induce the production of these cytokines involve MAPKinases (MAPK). Several MAPK signaling pathways can be activated as pJNK, p38 and ERK1/2. During *T. vaginalis* infection, it has been shown that the signaling pathways involved are ERK1/2, p38 and NF- κ B (An et al. 2002; Goral and Kovacs 2005). The activation of these signaling pathways are induced by various factors such as intercellular adhesion molecule type 1 (ICAM-1), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and also the association of various pathogens including *T. vaginalis* (J.-H. Chang, Park, and Kim 2006). A recent study has shown that the signaling pathways MAPK also lead to increased mRNA expression of toll-like receptors (TLR). In this study, the induction of mRNA and 9 TLR2.4 receptors are related to the activation of p38 (J.-H. Chang, Park, and Kim 2006). This work is interesting, but it is still necessary to confirm the increased expression of TLRs in epithelial cells, which could explain the maintenance of the infection in the long term by allowing the parasite to remain associated with the cells and prevent thus elimination through the vaginal flow.

Finally, it appears that the signal pathway are also MAPKs involved in the establishment of cell death in the form of apoptosis by activating Bcl-X_L (a Bcl-2 like) but not via the Bcl-2 pathway (J.-H. Chang et al. 2004) as well as NF- κ B in macrophages (J.-H. Chang, Park, and Kim 2006). Activation of apoptosis is related to the activation of the p38 pathway that leads to a release of cytochrome C inducing activation of caspase 3 and 9 (Chang et al, 2005).

Implementation of this apoptosis and autophagy may be demonstrated in epithelial cells to decipher more mechanisms related to the pathogenesis of the parasite.

2.4. Chemotherapy of uro-genital trichomoniasis

2.4.1. 5-Nitroimidazoles

Currently, 5-nitroimidazole is the only class of drugs commonly used for the oral or parenteral therapy of trichomoniasis. Among these drugs, only metronidazole and tinidazole are available in the USA and are authorized by the Food and Drug Administration (FDA) for the treatment of trichomoniasis.

Developed in 1959, metronidazole was approved in 1960s for the treatment of trichomoniasis and was the only drug with a cure rate approaching 100% with systemic treatment (Table 2) (Cosar and Julou 1959). Metronidazole is a small heterocyclic compound with a nitro group on the fifth position at imidazole ring. It is derived from the *Streptomyces* antibiotic azomycin. Metronidazole enters in *T. vaginalis* like an inactive molecule via passive diffusion. The drug itself is inactive, but it forms a cytotoxic nitro radical anion by anaerobic reduction through hydrogenosome using pyruvate-ferredoxin oxidoreductase (Chapman et al. 1985; Hrdý, Tachezy, and Müller 2008; Leitsch et al. 2014). This process is very sensitive to the presence of molecular oxygen, because oxygen is a strong competitor for ferredoxin-bound electrons. The nitro anion radical is hypothesized to bind transiently to DNA, disrupt or break the strand and cause the cell death (Lloyd and Kristensen 1985; Palermo and Dolores Mudry 2013). (Figure 2)

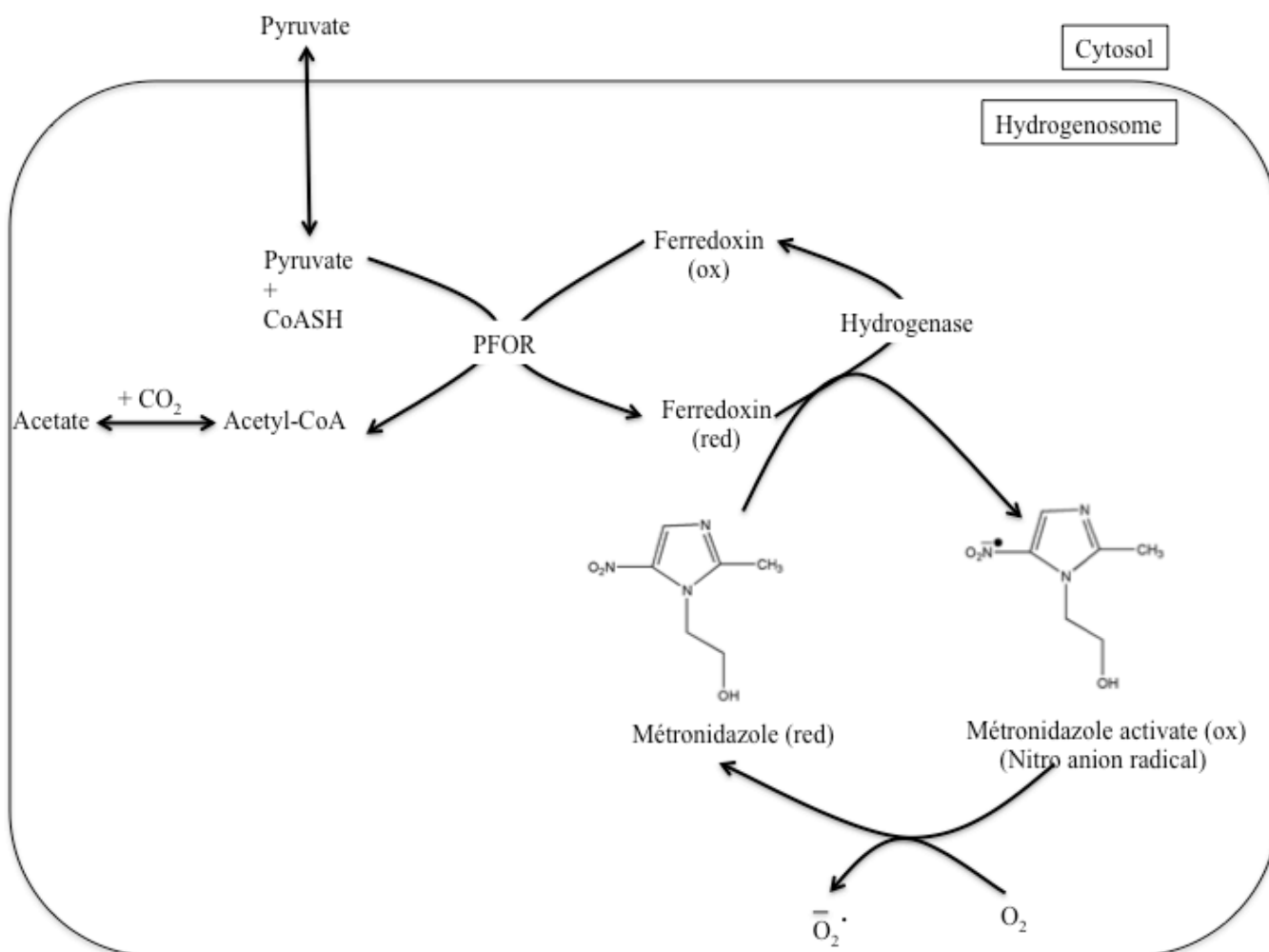


Figure 2 : Metronidazole activation in hydrogenosome.

Tinidazole is a second-generation nitroimidazole; it has a plasma elimination half-life twice that of metronidazole (12-14h vs. 6-7h, respectively) and is eliminated at a significant lower rate (Harp and Chowdhury 2011; Mattila et al. 1983). Tinidazole has a superior tissue distribution than those of metronidazole and the drug concentration found in the vaginal secretion are close to the levels found in serum. In consequence, it is delivered more effectively to the infected area than metronidazole (Joseph G. Lossick 1982). Tinidazole is curative at lower therapeutic doses than metronidazole and result in fewer and milder side

effects (J. G. Lossick 1990; Malla et al. 1988; Narcisi and Secor 1996). Its mode of action is similar to those of metronidazole.

In randomized clinical trials, the recommended oral metronidazole treatment resulted in cure rates of about 90% - 95% and treatment of tinidazole recommended resulted in cure rates superior of 90% (Harp and Chowdhury 2011). Randomised controlled trials comparing 2 g oral therapies of tinidazole or metronidazole show that tinidazole is equivalent or superior to metronidazole for the cure of trichomoniasis and resolution of symptoms (Forna and Gülmezoglu 2003). A study showed that for HIV infected women, a single dose of metronidazole is not efficient enough to treat trichomoniasis. In this case, a 7-day treatment with 500 mg metronidazole orally administered twice a day may be preferred (Kissinger et al. 2010). The single 2-g orally administered dose is usually favored because patient compliance is better and less than the total drug administered is necessary for healing. However, when the metronidazole dose is higher, side effect risk is more important. Furthermore, with a treatment duration of 7 days, patients are protected during this period from an immediate reinfection whereas this protection is not as reliable with a single dose unless the sexual partner(s) is treated simultaneously (Cudmore et al. 2004; J. G. Lossick 1990).

Metronidazole can also be administered intravenously, with a dose of 500 mg to 2 g administered over 20 min. Intravenous administration is rarely used but is associated with less severe side effects than oral dosing. Cure rates for oral and intravenous metronidazole treatment are similar (85 vs. 95%), and increase if the sexual partner(s) is treated simultaneously (J. G. Lossick 1990).

Although metronidazole intravaginal gel is the most effective drug among several topically applied antimicrobial, it is not recommended by the FDA because it is considerably less efficacious for treatment of trichomoniasis (>50%) than oral preparations of metronidazole (CDC 2010).

Despite the fact that the partners of patients with *T. vaginalis* infection are often infected too, high rates of asymptomatic infection means that they do not always seek treatment and they may re-infect their partner who had been treated. Sexual partners should be routinely treated (Kissinger et al. 2006; Workowski and Berman 2006).

Metronidazole is secreted in breast milk in small quantities, in consequence the treatment for lacting mother is a single 2 g dose followed by interruption of breast feeding for at least 24h. This recommendation is controverted because the small amount of drug exposure and the lack of evidence of risk to the neonate are poor reason to interrupt breast-feeding (Cudmore et al. 2004; J. G. Lossick 1990). Neonatal trichomoniasis is usually asymptomatic or mildly symptomatic and a spontaneous resolution of the condition is common before the sixth week of infant's life. If the infection is symptomatic or persists after six weeks, the infant may be treated with metronidazole as a single 50 mg/kg dose or a 10 to 30 mg/kg daily dose for 5 or 8 days (J. G. Lossick 1990).

Table 2: Topic and systemic treatment of urogenital trichomoniasis

Treatment	Route	Schedule	Recommendation
Metronidazole 2g	Oral	Single dose	First trimester of pregnancy and during lactation
Metronidazole 500mg	Oral	Twice daily for 7 days	Avoid consuming alcohol during treatment
Metronidazole 500mg	Pessarie (Flagyl [®]) or vaginal tablet (Tergynan [®])	10 days	
Metronidazole 500mg + nystatin 100 000 UI	Vaginal cream or pessarie (Flagystatin [®])	10 days	Cream formulated with nystatin for use against mixed vaginal infection due to <i>T. vaginalis</i> and <i>C. albicans</i>
Metronidazole 500 mg to 2 g	Intravenous	Single dose	Administered over 20 min
Tinidazole 2g (new)	Oral	7 to 10 days	A maximum of 4 g should not be exceeded during a 24-hour period

2.4.2. Side effects and hypersensitivity

Metronidazole is relatively cheap, effective, generally well tolerated, with patient suffering few or no side effect due to standard regimens. Common side effects include nausea, vomiting, headache, insomnia, dizziness, drowsiness, rash, dry mouth, and metallic taste, but these adverse reactions are usually mild. However occasional side effects and toxicity have been reported including eosinophilia*, leukopenia*, palpitation, confusion, convulsive seizure* and peripheral neuropathy*. These side effects have been found to be temporary and disappear after the cessation of the treatment (J. G. Lossick 1990).

* Cf definitions

Concerning patient with refractory trichomoniasis infection, the side effects due to metronidazole become a real problem. In fact, since metronidazole is the only treatment authorized for trichomoniasis in the USA, recurrent or resistant trichomoniasis are treated with increasing doses of the drug for longer periods. However, using higher doses, adverse reactions to metronidazole are much more frequent leading to patient intolerance, incomplete treatment courses and treatment failure (Akyol, Mungan, and Baltaci 2000; Ti, Lee, and Khoo 1996).

Nitroimidazole hypersensitivity is also an issue in the treatment of trichomoniasis and patient manifesting an immediate-type hypersensitivity reaction. In the event that this situation arises,

metronidazole desensitization, by oral or intravenous route and in consultation with a specialist is recommended (CDC 2010). A recent study shows that oral and intravenous metronidazole desensitization was 100% effective in the management of trichomoniasis in women with a significant nitroimidazole hypersensitivity reaction. Nonetheless desensitization was associated with minor side effects to metronidazole in 13% of women but was efficaciously managed with antihistaminic and steroid administration (Helms et al. 2008).

Metronidazole is a recommended treatment during pregnancy for bacterial vaginosis, nevertheless its use has been debated because the drug is known to cross the placenta. Therefore metronidazole as an FDA pregnancy category B group, has been contraindicated during the first trimester of pregnancy and is considered a second line of therapy in the latter stage of pregnancy (Cudmore et al. 2004). Similarly randomized trials comparing antitrichomonas agents during pregnancy have been realized by the Cochrane Pregnancy and Childbirth Group Trials with 842 pregnant women presented symptomatic or asymptomatic *T. vaginalis* infection. This study concludes that metronidazole, given as a single dose, is likely

to provide parasitological cure for trichomoniasis, but it is not known whether this treatment will have any effect on pregnancy outcomes (Gülmezoglu 2002). In fact, several trials using metronidazole in pregnant women for other reasons than trichomoniasis such as asymptomatic *T. vaginalis* infection or elevated fetal fibronectin levels have shown increased preterm birth rates (Andrews et al. 2003; Klebanoff et al. 2001). In addition, metronidazole has been reported to be mutagenic in eukaryotic systems (Celik and Aras Ates 2006; Dobiás et al. 1994; Lindmark and Müller 1976) and carcinogenic in rats that consumed daily dietary doses (Connor et al. 1977; Rustia and Shubik 1979). Although the scientific literature is still ambiguous, the potential toxicities in human have been voiced. However, the need for additional studies has been reported (Bendesky, Menéndez, and Ostrosky-Wegman 2002) and a recent retrospective analysis studying the risk associated with metronidazole treatment during pregnancy did not show association between taking drug during pregnancy and preterm birth, low birth weight, or congenital anomalies (Koss et al. 2012). A review on the treatment of vaginosis during pregnancy suggests that the treatment of asymptomatic *T. vaginalis* have no benefit for the prevention of preterm birth. Although it is not clear why treatment of *T. vaginalis* with metronidazole would be harmful, it has been speculated that dying *T. vaginalis* organisms elicit an inflammatory response or release a virus from the organism that increases the risk of preterm risk (Okun, Gronau, and Hannah 2005).

2.4.3. Resistance and treatment failure

An emerging problem with metronidazole is the increase of nitroimidazole resistance and particularly metronidazole resistance. Metronidazole resistance of *T. vaginalis* is defined as an aerobic minimum lethal concentration (MLC) greater than or equal to 50 µg/mL. Drug resistance to metronidazole has been reported between 2.5 and 9.6% of trichomoniasis cases

(Schmid et al. 2001; Jane R. Schwebke and Barrientes 2006). In addition, a recent study estimating the prevalence of *in vitro* aerobic metronidazole and tinidazole resistance from six US cities found that, among 538 *T. vaginalis* isolates, only 23 (4.3%) exhibit low level *in vitro* metronidazole resistance ([MLC] >50 µg/mL). No isolates exhibited moderate to high-level metronidazole resistance, suggesting that clinical resistance is unlikely to occur. In this study, MLCs of tinidazole are lower than those of metronidazole among *T. vaginalis* isolates and tinidazole resistance is not detected (Kirkcaldy et al. 2012). These results highlight the possibility that reliance on a single class of antimicrobial drugs for treating *T. vaginalis* infections may heighten vulnerability to emergence of resistance. In addition, in the absence of alternative to nitroimidazole treatment, cure can only be achieved by increasing dosages of metronidazole. However, side effects may limit the dose of metronidazole administration and sometimes necessitate stopping the treatment (Cudmore and Garber 2010).

Moreover, cases that cannot be treated with higher doses of metronidazole are a real challenge for both patient and practitioner. If treatment failure arise with a 2 g dose of metronidazole and reinfection has been excluded, the patient can be treated with 500 mg of metronidazole orally twice daily for 7 days. If this regimen fails, treatment with 2 g metronidazole or tinidazole orally once daily for 5 days should be considered (CDC 2010). Higher dosages were also used as 1 g tinidazole orally twice or three times per day together with 500 mg of tinidazole intravaginally twice a day for 14 days (J. D. Sobel, Nyirjesy, and Brown 2001).

Metronidazole resistance is not totally elucidated but it is assumed that it affects both aerobic and anaerobic mechanisms of parasite metabolism and it can be due to several mutations. In anaerobic resistance, PFOR activity decreases or disappears in *T. vaginalis* and the metabolism of the drug is disrupted. Moreover, the hydrogenase activity is impaired or

absent inhibiting the production of molecular hydrogen by hydrogenosome (Kulda 1999). In aerobic resistance, one transcription of the ferredoxin gene is reduced in aerobically resistant *T. vaginalis* strain (Quon, d' Oliveira, and Johnson 1992). However, the role of ferredoxin in metronidazole resistance is ambiguous. Some studies showed that interference of the ferredoxin gene did not lead to *in vitro* metronidazole resistance (Land et al. 2004) while other study demonstrated that ferredoxin is involved in nitroimidazole susceptibility (Vidakovic et al. 2003). Moreover, a decrease of hydrogenase activity leads to the reduction in hydrogen production and oxygen-scavenging pathway is affected. Oxygen being a highly efficient electron receptor, if the level of oxygen is increased in the hydrogenosome, the reduction and the activation of metronidazole are reduced (Cudmore et al. 2004). A recent study showed that flavin and particularly the flavin reductase 1 (FR1), formerly known as NADPH oxidase, plays a role in the reemergence of metronidazole resistance in *T. vaginalis*. In fact, the FR1 is not expressed in highly metronidazole-resistant strains and the transfection of a plasmid bearing a functional FR1 gene nearly completely restored metronidazole susceptibility (Leitsch et al. 2014).

2.4.4. Clotrimazole

During pregnancy, the Center of Disease Control and Prevention recommended a single 2-g dose of metronidazole. Alternatively, a daily dose of 100 mg of clotrimazole can be delivered intravaginally at bedtime during 6 days and can temporary relieve the woman in the first trimester. A cure rate of 50% is achieved with this method, but if a systemic treatment is necessary, the standard metronidazole treatment is given during the second or the third trimester (Gülmezoglu 2002).

In addition, Krieger et al. tested several *T. vaginalis* strains in a time-kill assay against clotrimazole and found MICs greater than 100 µg/ml for 90% of the isolates, although several strains were susceptible (Krieger, Dickins, and Rein 1985). Another in vitro study showed that all five strains tested were resistant to clotrimazole, and MICs were >100 µg/ml (Sears and O'Hare 1988).

2.4.5. Alternative potential drugs

Although the 5-nitroimidazole compounds are the most effective drugs to treat *T. vaginalis* infection, disulfiram and nithiamide may be useful to treat patients with hypersensitivity to 5-nitroimidazole drugs. Moreover, albendazole and coenzyme B12 may be helpful in combination with metronidazole or tinidazole for treatment of patients with highly resistant *T. vaginalis* infection (Goodhew and Secor 2013).

In addition, in vitro studies show that the IC₅₀ for albendazole against *T. vaginalis* were 13.2 µg/ml (50 µM) and 0.899 µg/ml (3.39 µM) respectively after 4 h or 48h exposure. (Navarrete-Vázquez et al. 2003; Oxberry, Thompson, and Reynoldson 1994). Moreover a study shows that albendazole and mebendazole have been found effective *in vitro* against *T. vaginalis* (Sears and O'Hare 1988).

In case of nitroimidazole allergy and when desensitization is not possible, treatment options are limited. These alternative treatments include intravaginal preparations of paromomycin (Nyirjesy et al. 1998), boric acid (Muzny, Barnes, and Mena 2012), furazolidone (Goldman et al. 2009), nonoxynol-9 intravaginal suppositories (Livengood III and Lossick 1991) and povidone–iodine (betadine) douches (Wong, Wilson, and Chew 1990) but all are of limited efficacy. In addition, the use of nonoxynol-9 was gradually abandoned

because of its toxicity (Van Damme et al. 2002; Richardson BA 2002; Roddy RE et al. 2002; Wilkinson 2002) and studies have shown that nonoxynol-9 resulted in a rupture of the vaginal epithelial barrier and accelerated HIV replication (Van Damme et al. 2002; Van Damme et al. 2008).

In the case of treatment failure with metronidazole and tinidazole due to drug resistance, alternative intravaginal therapies exist, although their efficiency is limited and only ensures anecdotal healing. Intravaginal paromomycin cream could be considered, but this treatment is very irritating to the vaginal mucosa and some patients developed vaginal ulcerations that resolved spontaneously (Nyirjesy et al. 1998). Furthermore, a case report showed that drug resistant trichomoniasis can be treated successfully with a combination therapy using paromomycin intravaginal cream administered nightly with oral tinidazole dosed at 1 g three times a day for 14 days (Nyirjesy, Gilbert, and Mulcahy 2011). A second case report showed that vaginal acidification with intravaginal boric acid used for 1 and 5 months, respectively, in two patients who tolerated this therapy, resulted in resolution of recalcitrant *T. vaginalis* (Aggarwal and Shier 2008).

A study described that nitazoxanide, a 5-nitrothiazolyl derivative, has an *in vitro* activity against *T. vaginalis*. Nitazoxanide exhibited IC₅₀ and IC₉₀ values of 0.034 and 2.046 µg/ml against *T. vaginalis* (Cedillo-Rivera et al. 2002). However in 2007, there was a treatment failure with nitazoxanide to cure trichomoniasis in three women presented nitroimidazole resistance (Dan and Sobel 2007).

Table 3: Drugs chemicals formulas

Molecules	Chemical formulas
Metronidazole	
Tinidazole	
Clotrimazole	
Disulfiram	
Nithiamide	
Mebendazole	
Paromomycin	
Furazolidone	
Povidone-iodine	
Nitazoxanide	

2.5. Prevention and strategy for vaccines

Other than total abstinence, condom use remains the best and the most reliable protection against sexually transmitted infections. Condoms must be used consistently and correctly to be effective. However some women, mostly in developing countries have no choice to use condoms principally for religious or cultural reasons.

With an increase incidence of nitroimidazole-refractory strains and the lack of a safe and alternative to oral nitroimidazoles, disease treatment and prevention using local intravaginal formulation or vaccine clearly are desirable.

The development of a vaccine that could provide long-term protection against *T. vaginalis* infection would solve many of the issues that currently undermine efforts of control. Vaccination against trichomonad species is already in use commercially. In fact, it already exist a vaccine administered subcutaneously against *Tritrichomonas foetus* which is a flagellate protozoan similar to *T. vaginalis* that infects cattle. Given the similarity between *Tritrichomonas foetus* and *T. vaginalis*, development of a vaccine against human trichomoniasis seems both achievable and with the potential for huge financial, social, and health impact globally (Cudmore and Garber 2010).

However the vaginal way seems to be a really good alternative for the prevention and the treatment of *T. vaginalis* infections.

3. Vaginal physiology as basis for new approaches in prevention and treatment of vaginal trichomoniasis

3.1. Vaginal physiology

The vagina is a tubular, fibromuscular organ that extends from the cervix of the uterus to the vaginal vestibule measuring in length about 9 cm (Van de Graaff 2001). Its high elasticity and relaxation of the wall allow it to extend to the vicinity of the uterus during delivery. It is situated in the pelvic cavity, in front of the rectum, back of the bladder and urethra. It receives the penis during intercourse and serves as a gateway to menstrual flow and baby during childbirth. The deepest portions of the vagina called the fornix of the vagina surround the cervix (Tortora 2009).

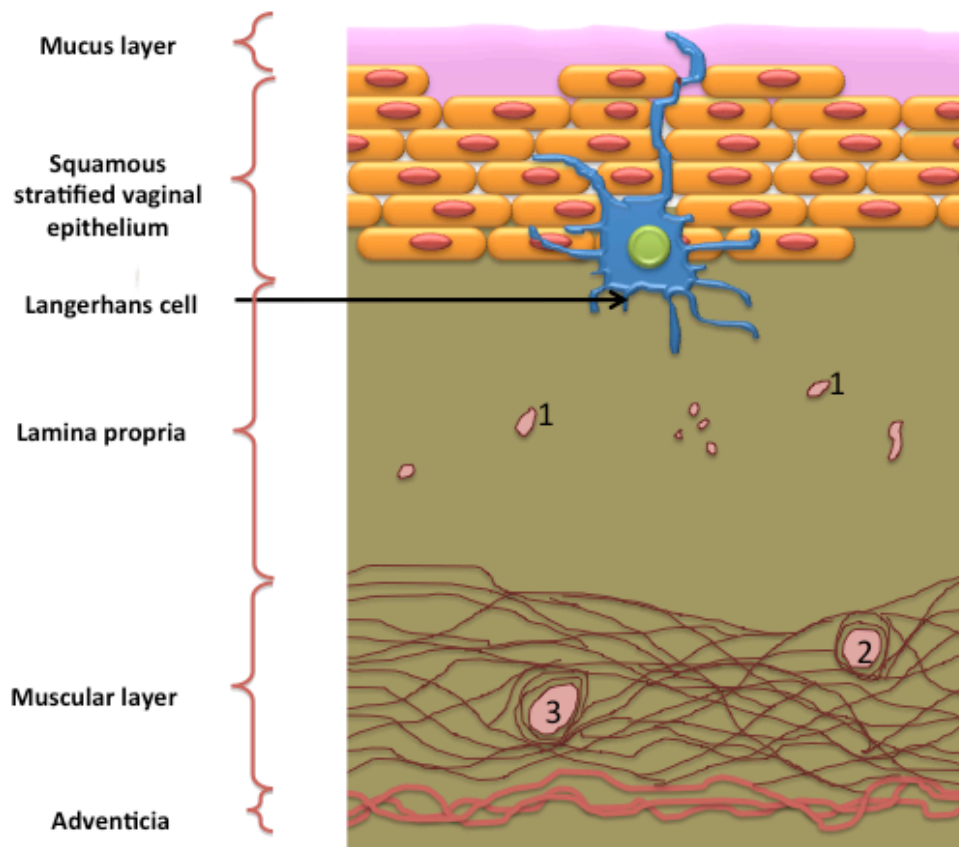


Figure 3 : Schematic drawing of the mucosa
 1 : capillary vessels ; 2 : artery ; 3 : vein.

The vaginal wall consists of three layers: the epithelial layer, the muscular coat and adventitia (Figure 1). The vagina has an excellent elasticity because of the smooth elastic fiber in the muscular coat. Loose connective tissue of adventitia further increases the elasticity because of the vaginal wall (Hussain and Ahsan 2005). A cell turnover of about 10–15 layers is estimated to be in the order of 7 days. The epithelium is a non-cornified and stratified squamous epithelium. The thickness of the vaginal epithelial cell layer changes by approximately 200 – 300 μm depending of the age and the period in the menstrual cycle. The state of the epithelium is highly dependent on steroid hormones and it thickens with hormonal activity. In the first part of the cycle, the epithelium has a proliferative activity accompanied

by a significant glycogen synthesis. After ovulation, glycogen synthesis is slowed down, the cells desquamate and epithelium thickness decreases (Sjöberg, Cajander, and Rylander 1988).

The vaginal mucosa form a series of transverse folds often called rugae that provide distensibility, support and increase its surface. It is lined with a viscoelastic gel called vaginal mucus compound of organic and inorganic salts, mucin proteins including immunoglobulins, carbohydrates, urea and fatty acids (Vermani and Garg 2000). The mucus protects the vaginal mucosa from the external environment and ensures lubrication (Junginger, Thanou, and Verhoef 2002). Its composition and physical characteristics vary according to the menstrual period. It plays an important role in the physiological activity of the vagina, including the regulation of sperm transport, sperm reservoir, sperm energy source, water balance, anti-microbial activity and also the absorption and action of drugs (Khanvilkar, Donovan, and Flanagan 2001). It would be capable of capturing foreign particles and remove them. By contrast, some particles could be directed to the uterus. This is the case, for example for sperm during ovulation (Tang et al. 2009).

The vagina is highly vascularized mainly by uterine artery, vaginal artery and hemorrhoidal artery average. The arteries, blood vessels and lymphatic vessels are abundant in the vaginal wall (Hussain and Ahsan 2005). The fact that the vagina is surrounded by large venous plexus plays a very important role in absorption including drugs which are susceptible to hepatic metabolism. Thus, a substance absorbed from the vagina does not undergo first-pass metabolism because the blood leaving the vagina enters the peripheral circulation via a rich venous plexus, which empties into the main internal iliac veins (Richardson and Illum 1992).

The vagina does not possess gland, however it secretes a large amount of fluid (Paavonen 1982). Vaginal fluid is composed of secretion and transudations from blood vessels, cells

derived desquamation of the vaginal epithelium, leukocytes, secretions of the endometrium and fallopian tubes but also menstrual secretions and sperm (Paavonen 1982; Burgos and de Vargas-Linares 1978; Lentz 2013). Like the thickness of the vaginal epithelium, the amount and composition of vaginal fluid is changed during the menstrual cycle. Vaginal fluid is approximately evaluated to be between 0.5 and 0.75 g present in the vagina at any moment. (Owen and Katz 1999; Owen and Katz 2005) The human vaginal fluid may contain enzymes, enzyme inhibitor, proteins, carbohydrates, amino acids, alcohols, hydroxyl-ketones and aromatic compound. (Wagner and Levin 1978) Sexual arousal may change the composition and the quantity of vaginal fluids (Preti, Huggins, and Silverberg 1979; Wagner and Levin 1978; Levin and Wagner 1977) and that can change the drug release in the vagina. (Deshpande, Rhodes, and Danish 1992; Vermani and Garg 2000; Yu et al. 2011)

The vaginal pH is normally acidic (approximately 4 - 4.5) and usually varies between 3.5 and 5. This value is maintained by the resident flora and mainly by Lactobacilli (in particular *Lactobacillus acidophilus vaginalis* also called Döderlein bacilli) that convert glycogen from exfoliated epithelial in lactic acid and product other fatty acids. However, this is not necessarily the primary source of low vaginal pH; fatty acids, including lactic acid, produced by vaginal epithelial cells and released into the secretions are probably a more important source (Preti, Huggins, and Silverberg 1979). Lactobacilli also produce bactericidal substances such as hydrogen peroxide and surfactants that play a role in preventing infections (Boris and Barbés 2000). The low vaginal pH appears as the primary mechanism in controlling the composition of microbiota. In fact, *in vitro* studies have shown that acidification by lactobacilli growth can inhibit the proliferation of pathogen microorganisms, such as *Candida albicans* (Young, Krasner, and Yudkofsky 1956), *Escherichia coli* (Reid et al. 1988), *Gardnerella vaginalis* and other bacteria cultured from vaginal specimens obtained

from women with bacterial vaginosis. The menstrual secretions, cervical mucus and sperm can raise the vaginal pH (Woolfson, Malcolm, and Gallagher 2000).

The pH plays a role in the amount of drug absorption and must be taken into account in the formulation of drug delivery systems. An increase of the absorption of leuprolide (a GnRH analog) was observed after adding organic acids which partially dissolve the cellular cement (Okada et al. 1983). In addition, the efficacy of a gel contained dinoprostone (prostaglandin E₂) was significantly related to the pH of the vagina (Ramsey et al. 2002).

In postmenopausal women, the physiology of the vagina undergoes major changes as a progressive atrophy of the vaginal epithelium, an increase of the pH (6.0-7.5) and a decrease in the amount of vaginal secretions (Caillouette et al. 1997). These features can significantly change the characteristics of a drug delivery system.

3.2 Drug delivery systems for vaginal administration

The anatomical position, the rich vascularization and the large size of the vagina make it an ideal candidate for the local or systemic drug application (Weber, Desai, and Darbyshire 2005; Vermani and Garg 2000; Richardson and Illum 1992).

Thus, the vaginal route has many advantages for drug delivery. It allows drugs to avoid hepatic primary channel and this has been demonstrated with propranolol (a sympatholytic non-selective beta blocker) which has a better bioavailability after administration by the vaginal route than by orally (Patel, Warrington, and Pearson 1983). There is also a decrease of side effects observed by vaginal administration of bromocriptine, a dopamine agonist, compared to the oral administration (Vermesh, Fossum, and Kletzky 1988). Similarly, hepatic

side effects induced by hormone replacement therapy or birth control are greatly reduced (Ceddars and Judd 1987). The vaginal also overcomes the disadvantages associated with the pain, tissue damage and infection may occur by other parenteral routes. Furthermore, the vaginal route enable the self-insertion and dosage form modification (Okada et al. 1983).

However, it is important to note that vaginal physiology is very particular (vaginal fluid, pH mucus, bacterial flora) and variable according to the age, the phase of the menstrual cycle or during sexual activity. In addition, the enzymes present in the vagina are the prominent barriers for the absorption of intact peptide and protein drugs into the systemic circulation. In fact the most of exopeptidases and endopeptidases that digest the peptides and proteins are present in the vaginal epithelium (Chien and Lee 2013). All these factors must be taken into account in the formulation of topical formulation for the vaginal route.

Numerous studies have shown a good permeability of vaginal mucosa to a wide range of compounds including peptides and proteins. The vaginal route offers a good alternative to the parenteral route for some drugs (Valenta 2005). However, the vaginal route is traditionally used for the local delivery of active substances such as antibacterial, antifungal, antiviral, anti-inflammatory or spermicides and steroids. In the last decade, major advances have been noted in the field of antimicrobial agents as compounds or formulations prevent the transmission of STI including AIDS.

Ideally, a topical antimicrobial formulation for the vaginal route should have the following requirements:

- Once administered, the formulation must easily line the vaginal mucosa. Ideally, it will have to form a barrier (physical and / or chemical) capable of preventing the interaction of the

infectious agents with the vaginal mucosa;

- it must respect the normal acid vaginal pH and normal vaginal flora;
- it must remain in place as long as possible to interact with infectious agents before and especially after sexual intercourse;
- it must retain its rheological and mucoadhesive properties after dilution by physiological fluids such as vaginal fluid. Indeed, it is known that formulations administered in the vagina are diluted by vaginal fluid. This dilution can cause a decrease in efficacy of the formulation by the loss of gelling, decreased mucoadhesive properties and the feeling of discomfort along with its removal of the vaginal cavity;
- microbicide activity of the formulation should be preserved;
- it must be not toxic;
- it must maintain the integrity of condoms;
- it must be colorless, odorless and have acceptable taste and flavor;
- it must be fast-acting, long-lasting, non-irritating and undetectable to either partner;
- it must be inexpensive to manufacture and purchase and available without a prescription;
- it must be safe to use more than once a day and for long periods of time;
- it must be effective against multiple STDs including HIV/AIDS;
- it must be available in contraceptive and non-contraceptive forms.

3.3 Vaginal formulations for *T. vaginalis* treatment

A formulation against vaginosis and particularly *T. vaginalis* must obviously be appropriately formulated for vaginal administration.

In summary, it must be inexpensive, colorless, odorless, tasteless, and non-contraceptive, easy to use, can enable various modes of operation and should not interfere with sexual pleasure (Buckheit Jr. et al. 2010).

Dosage forms for vaginal administration are various: liquids as solutions, emulsions and vaginal suspensions, semi-solid as creams, ointments and gels,, or solid as pessaries, vaginal tablets, vaginal capsules and vaginal films (Vermani and Garg 2000). Recently, vaginal rings have been devised for the therapy and contraceptive hormone release (van den Heuvel et al. 2005; Lopez et al. 1996; Roumen et al. 2001). There are also other dosage forms such as semi-solid including creams, ointments and gels. Vaginal rings, unlike the semi-solid forms formulated for line the vaginal mucosa, are positioned at precise place in the upper third of the vagina (particularly susceptible to pathogen infection) near the cervico-vaginal junction. They may liberate drug in a controlled way and during a long time (up to several months) in the lumen of the vagina. Vaginal rings generally constituted of a polymeric matrix (silicone, thermoplastic materials ...) containing drugs (Malcolm et al. 2010; Ursell et al. 2014).

Ideally, the choice of vaginal drug administration depends on the applicability of the intended effect. When a local effect is required, semi-solid or fast dissolving solid system will be used. For a topical effect, generally, a bioadhesive dosage form or intravaginal ring system would be more preferable (Hussain and Ahsan 2005).

For *T. vaginalis* treatment, nitroimidazoles are already formulated for topical application in the vagina. Metronidazole vaginal tablets containing 100 mg of metronidazole were compared with vaginal tablets containing placebo and conventional oral metronidazole treatment consisting of two 500 mg doses daily for 7 days. The cure rate was significantly different between the placebo group and the two metronidazole groups, but no significant difference in clinical efficacy was observed between the group treated orally and the group treated with the bioadhesive carrier (Bouckaert et al. 1995). Furthermore, a cure rate of 64% was obtained after an administration of a vaginal tablet containing 100 mg of metronidazole (Voorspoels et al. 2002). A recent study showed that a high-dose intravaginal metronidazole combined with miconazole offers the possibility of a well-tolerated treatment that avoids the systemic adverse effects of nitroimidazoles for the treatment of trichomoniasis (Jane R. Schwebke, Lensing, and Sobel 2013). Other topical agents, including mebendazole, paramomycin, or nonoxynol-9 creams have been used with limited success (Beigi 2012). Actually, topically-applied treatments should be limited to adjunctive therapy or particular cases of allergy or resistance. Nevertheless some data have shown that although research is needed to define the optimal dose of the active product in vaginal formulation and that further refinement is required before the process can be put into practice, treatment of trichomoniasis by vaginal route can be a real alternative to systemic treatment.

Recently, different approaches to encapsulate drugs in nanoparticles have been proposed (Arias et al. 2010; Elzatahry and Eldin 2008; Lages et al. 2013). This strategy would have different purposes, the use of nanoparticles could reduce an eventual toxic effect of the drug while promoting the targeting site of action. Nanoparticles can also increase the apparent aqueous solubility of hydrophobic drugs (Esposito et al. 2013) and could protect fragile molecules before and after administration. Moreover, unloaded nanoparticles can have an activity against pathogen (Lherm et al. 1987). The objective being that nanoparticles are able

to cross the physiological barrier constituted by the mucus layer and reach the epithelium. However their ability to reach vaginal epithelium and/or diffuse locally could be a limit to this concept. Indeed, it has been shown that unloaded polystyrene nanoparticles were able to diffuse through the physical barrier constituted by the vaginal mucus (Lai et al. 2007; Lai, Wang, and Hanes 2009; Lai et al. 2011; Tang et al. 2009). Nevertheless, the drug release is certainly less important than that of small molecules in solution. However, the modulation of the particle surface by techniques such as pegylation or thiolation could help to improve their ability to diffuse through the mucus.

Finally, the semi-solid drug delivery forms such as gels are widely used in the development of topical formulation against pathogen of the vagina (das Neves and Bahia 2006). These forms are reviewed in the following paragraph.

3.4. Pharmaceuticals gels

Most pharmaceutical gels contain hydrophilic polymers that allow gelation mechanism. Thanks to their high water content, these formulations are primarily intended to be applied on mucous membranes (oral, nasal, ophthalmic, rectal or vaginal) or on the skin for cosmetic or therapeutic use.

The advantages of semi-solid preparations are their tolerance, their feasibility and their low cost of production. Moreover, hydrogels are generally colorless, odorless, tasteless and can be used in combination with vaginal devices or condoms (Garg et al. 2010; Pilcher 2004). However, different disadvantages need to be considered because these vaginal dosage forms can be messy to apply, uncomfortable, sometimes embarrassing when they leak into the undergarments and the need to use an applicator for administration (Garg et al. 2010; Hussain

and Ahsan 2005). Indeed, one of the problems presented by conventional drug delivery systems is the rapid detachment of the application site (Garg et al. 2003). For this reason, gels are the most adapted pharmaceutical form for the vagina allowing thus to overcome these drawbacks.

Gels are semi-solid substances consisting of a small amount of a solid dispersed in a greater proportion of liquid and having both the characteristics of a solid and a liquid. These systems form a three dimensional polymeric matrix with a high degree of crosslinking. They consist of long chains that are connected together at specific points, but the connections must be reversible. Gelation mechanisms are still poorly understood, but researchers are trying to develop and improve molecules with these properties. Gels also have several advantages over other drug delivery systems such as higher bioavailability, a higher degree of safety, versatility and low cost (Justin-Temu et al. 2004; das Neves and Bahia 2006). The pharmaceutical gels consist in natural polymers, in particular some proteins (collagen, gelatin) and polysaccharides (alginate, carrageenan, guar gum), or semi-synthetic polymers (carboxymethylcellulose, hydroxypropyl methylcellulose) or synthetic (carbomer, pluronic[®]) or inorganic substances (aluminum hydroxide, bentonite, laponite) (Ofner and Klech-Gelotte 2013).

Pharmaceutical gels must have different properties such as stability in acidic medium, adhesion, non liquefaction at body temperature, slowly dissolving, be lubricant, non-greasy and non-irritating (Ofner and Klech-Gelotte 2013). Moreover, when gels are formulated for the vagina, other elements should also be taken into account. In fact, the gel may **(i)** maintain an optimum pH for vaginal epithelium, **(ii)** facilitates the application to obtain a homogeneous distribution of the drug, **(iii)** be retained into the vagina, and **(iv)** be compatible with other co-

administered substances (Chien and Lee 2013). Gels can also be used as controlled-release devices.

In terms of their effectiveness, the most important problem presented by these forms is certainly their low remanence to the epithelial surface and their rapid detachment from the application site (Garg et al. 2003; Vermani and Garg 2000). In order to overcome this problem, mucoadhesive polymers are often added to formulations for immobilizing the gel as long as possible to the vaginal mucosa (Justin-Temu et al. 2004).

For all these reasons, thermosensitive and mucoadhesive gels are very interesting for the prevention and the treatment of vaginal pathogens such as *T. vaginalis*. Thus, they can present the property of being liquid at low temperature, facilitating thus the spreading of the formulation on the whole vaginal area even in most inaccessible parts and move into a semi-solid state at the temperature of the body to easy their remanence.

In the sections below, we discuss in general the hydrogels based on thermosensitive polymers and/or mucoadhesive gels used for vaginal administration. These gels may have antiparasitic, antibacterial, antifungal or antiviral activity.

3.4.1 Mucoadhesive hydrogels

Bioadhesion can be defined as the state in which two materials, at least one of which is biological in nature, are held together by interfacial forces for some time. When this phenomenon is due to the coating of mucus, it is called mucoadhesion (das Neves and Bahia 2006). First of all, it must be remembered that the vaginal epithelium is not strictly a mucosal epithelium, as it possesses no mucus producing goblet cells. Thus, mucoadhesion is an

appropriate process to consider. In this respect, gels are formulated by using mucoadhesive materials (Justin-Temu et al. 2004). The target of mucoadhesive excipients is the mucus that is highly viscous and forms a protective layer on the outer wall of the organs.

During a long time, the bioavailability of many drugs has been altered due to the neglect of this phenomenon. Ponchel et al. have developed formulations for oral route taking into account this problem (Ponchel and Irache 1998; Ponchel et al. 1997). In order to circumvent it, they used mucoadhesive polymers that allowed to increase the drug residence time on the gastrointestinal mucosa. The vaginal mucosa is also favorable to this phenomenon because it is coated with a thin layer of mucus. The vaginal route is like the intestinal tract, well arranged for mucoadhesion. Gels are the most studied forms for mucoadhesive systems for vaginal administration (Edsman and Hägerström 2005). Moreover, excellent bioadhesion could increase the residence time of a drug at the absorption site by interacting with the mucosa (Duchêne and Ponchel 1992).

Several theories have tried to explain mucoadhesion, but the theory of interpenetration is the most recognized one. Mucoadhesion takes place in two steps: creation of intimate contact between the dosage form and the mucosa, and interpenetration of the components of both systems. In the case of a gel system, the chain ends and smaller molecular weight chains contribute to the interpenetration process (das Neves and Bahia 2006). Furthermore, an adequate solubility of the bioadhesive gel in the mucus is essential for good mucoadhesion (Huang et al. 2000).

Mucoadhesives are synthetic or natural polymers that interact with the mucous layer covering the mucosal epithelial surface and mucin molecules constituting a major part of the mucus.

The polyacrylic acid (PAA also called carbomer or carbopol[®]) is a bioadhesive polymer with a high molecular weight, known for his adherence to the mucosal hydrated cells of eyes, nose, lungs, gastrointestinal tract and vagina (Dittgen, Durrani, and Lehmann 1997). The polymers of PAA are pH sensitive. In fact, their ionization depends on the pH changes, and when pH increases above the pKa of the carboxyl groups, they form a gel. PAA gel is very used in many formulations already on the market for vaginal applications. It is found, for example in the following specialities: Rozagel[®] (called also Flagy gel[®]) and Rozex[®], which are antibacterial gels containing metronidazole combining a Carbopol[®] 940 for the first and Carbopol[®] 980 for the second, and a vaginal moisturizer, Replens[®], consisting in polycarbophil and Carbopol[®] 974P. Vaginal-Metrogel[®] is a Carbopol[®] 940 gel containing 0.75% of metronidazole that have already shown an activity against *T.vaginalis* after vaginal administration but its activity is limited in comparison with oral delivery (Jack D. Sobel et al. 2006; Wain 1998). The PAA is also used in antimicrobial formulations developed for the prevention of HIV transmission. This was the case of BufferGel[®] containing Carbopol[®] 974P and VivaGel[®] containing Carbopol[®] 971P (Mumper et al. 2009).

Recently, thiolated polymers have been studied as new mucoadhesive molecules. In a comparative study, these polymers have shown a longer adhesion time and an adhesion work more important than those of the polymers frequently (Valenta 2005). This is due in part to disulfide bonds that form with the mucin present in mucus (Grabovac, Guggi, and Bernkopschnurch 2005).

Other polymers such as chitosan, carrageenan and sodium alginate are interesting for mucoadhesive formulations (Valenta 2005).

Chitosan is a promising bioadhesive material at physiological pH. This polymer has properties considered to be essential for mucoadhesion, including the presence of OH and

NH₂ groups that are able to make hydrogen bonds. Moreover a study showed the possibility to use chitosan, in different proportions, with anionic polymers for the preparation of mucoadhesive tablets used for vaginal metronidazole delivery (El-Kamel et al. 2002). In addition, the mucoadhesive properties of chitosan can be greatly improved, while maintaining its biodegradability, if thiol groups are added (Bernkop-Schnürch, Guggi, and Pinter 2004).

3.4.2 Thermosensitive hydrogels

Hydrogels are considered like a three dimensional grid that have the ability to absorb a large amount of water and are insoluble in water at the temperature, the pH and the physiological ionic strength. Thanks to this remarkable property that gives them a good biocompatibility, they are used in a wide range of medical applications such as implants, contact lenses and controlled release systems (Graham and Wood 1982).

Environmentally-sensitive gels are systems that can dramatically alter their physical characteristics as a result of exposure to environmental changes. Of particular importance to the vaginal administration are thermosensitive gels. These gels are systems capable of gelling in response to temperature change, generally from ambient to body temperature (das Neves and Bahia 2006). Usually, the gelation temperatures have been considered to be suitable if they are in the range of 25–37°C. The thermoreversible properties of these gels may be evaluated by rheological parameters such as the temperature of sol-gel transition modules and viscoelasticities (J. Y. Chang et al. 2002).

Different sorts of thermosensitive polymer exists such as cellulose derivatives, chitosan with polyols salt, *N*-isopropylacrylamide copolymer, the poly(ethylene oxide) / poly (D, L-lactic acid-co-glycolic acid) PEO / PLG and the poloxamer gels.

Poloxamers (Pluronic[®], Lutrol[®]) are A-B-A type triblock copolymers composed of units of polyethylene oxide (PEO) and polypropylene oxide (PPO) with a structure type: POE-POP-POE. The concentrations of aqueous poloxamers give thermoreversible gels. The phenomenon thermogelation is perfectly reversible and is characterized by a transition temperature sol-gel (T_{gel}). Below this temperature, the sample remains fluid and above the T_{gel} , it becomes semisolid. The thermogelation results in interactions between the various segments of the copolymer (Gilles Dumortier et al. 2006). With the increase of the temperature, the monomer components in the poloxamer micelle aggregate with the hydrophobic block of dehydration POP. If the temperature is increased further, the micellization is followed by gelation for relatively concentrated samples. Various structures of gelation was observed (G. Dumortier et al. 1991; Gilles Dumortier et al. 2006). Poloxamer gels extend the residence time at the site of application that improves the bioavailability and efficacy of the drug and their toxicity is limited (Johnston and Miller 1985).

4. Conclusion

Currently, the fight against *T. vaginalis* infections is to act on both curative and preventive levels. In order to reach these objectives, a strategy consists in developing mucoadhesive forms able to line the mucous colonized by the parasite. The persistence of the active ingredient to the surface of the mucosa should be as long as possible. In this context,

thermosensitive and mucoadhesive hydrogels containing metronidazole seem to be an interesting alternative to systemic treatments.

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TRAVAUX EXPERIMENTAUX

Chapitre 1: Drug-free hydrogel-containing poly(isobutyl cyanoacrylate) nanoparticles: Thermal analysis and in vitro evaluations of trichomonacidal activity and toxicity using cell models and pig vaginal mucosa

Drug-free hydrogel-containing poly(isobutyl
cyanoacrylate) nanoparticles: Thermal analysis and *in*
vitro evaluations of trichomonacidal activity and
toxicity using cell models and pig vaginal mucosa

Bénédicte Pradines^{a,b}, Vanessa Lievin-Le Moal^b, Christian Bories^b, Christine Vauthier^a, Madeleine
Djabourov^c, Philippe M. Loiseau^b, Kawthar Bouchemal^a

^a Institut Galien Paris Sud, UMR CNRS 8612, School of Pharmacy, Univ Paris-Sud, France

^b Antiparasitic Chemotherapy, UMR CNRS 8076, Faculty of Pharmacy, Univ Paris-Sud, France

^c Laboratoire de Physique Thermique, ESPCI-ParisTech, France

Abstract

Micelle formation was followed by micro-DSC and rheology for aqueous solutions of pluronic[®] F127 containing or no nanoparticles (NPs) of poly(isobutylcyanoacrylate) (PIBCA). The presence of NPs prevents the total thermoreversibility of the hydrogel. Moreover, the NPs were degraded during the micellisation and a formation of mixed micelles containing PIBCA was suspected. The toxicity of the NPs was evaluated using different cell models: HeLa cells as undifferentiated and non-oriented cells derived from the human cervical carcinoma, Caco2/TC7 as differentiated and oriented cells from human colorectal adenocarcinoma characterized by the presence of the tight junctions, and HT-29/MTX as human colon adenocarcinoma cells that are differentiated and oriented cells characterized by the presence of the tight junctions and the production of mucus. The NPs showed an important toxicity against HeLa cells but no significant toxicity was observed against Caco-2/TC7 and HT-29/MTX. Histological experiments showed no toxicity against pig vaginal mucosa. Finally, the PIBCA NPs showed a very important trichomonacidal activity.

Keywords: Nanoparticles, isobutylcyanoacrylate, pluronic[®] F127, thermosensitive, hydrogel, *Trichomonas vaginalis*, HeLa cells, HT-29/MTX, Caco-2/TC7.

1. Introduction

Trichomonas vaginalis is a protista parasite responsible for the uro-genital trichomoniasis, one of the most common sexually transmitted disease (STD) in the world. This disease has important medical, social, and economical implications. Trichomoniasis is therefore reported to be a major cause of pathology in obstetrics and gynecology. Reports have implicated *T. vaginalis* in upper reproductive tract post surgical infections, reversible infertility, premature rupture of the placental membranes, premature labor, low-birth-weight infants, neonatal morbidity and mortality (Cotch 1990; Cotch et al. 1997; Hardy et al. 1984; Minkoff et al. 1984).

It has been reported that *T. vaginalis* causes discomfort and psychosocial distress in infected patients. The symptoms include a discharge that is usually copious and frothy, resulting in local pain and irritation. Pruritus might be present. Symptoms often peak just after menses. As demonstrated with other STDs characterized by vaginal inflammation, there is an increased rate of the Human immunodeficiency virus (HIV) acquisition associated with *T. vaginalis* (Laga, Nzila, and Goeman 1991) The HIV seroconversion in female prostitutes was significantly associated with the occurrence of other nonulcerative STDs, such as trichomoniasis. Certain factors such as poor personal hygiene, multiple sex partners, low socio-economic status and underdevelopment are associated with high incidence of the infection.

In this purpose, the present work aimed to combine the effect of hydrogels and nanoparticles for the design of new vaginally-applied microbicide formulation for the treatment of trichomoniasis. Since unloaded poly(isobutylcyanoacrylate) (PIBCA) nanoparticles had been described to exhibit antiprotozoal properties (Lherm et al., 1987), we hypothesized that they could have an activity against *T. vaginalis*. In addition, the

nanoparticles can be used as a vehicle and tank for drugs against *T. vaginalis*. Before adding any drug to this formulation, we proposed to disperse mucoadhesive PIBCA nanoparticles (NPs) coated with thiolated chitosan into a thermosensitive hydrogel composed of pluronic[®] F127. Thiolated chitosan was used because we have already shown its capacity to improve mucoadhesion (Petit et al. 2012).

Pluronic[®] F127 is an amphiphilic triblock copolymers with block compositions EO₉₈PO₆₉EO₉₈, (where EO refers to ethylene oxide and PO to propylene oxide). The role of this hydrogel is to act as a vehicle for the NPs and/or a physical barrier to *T. vaginalis* infection in the case the formulation would be used for preventing therapy. The thermosensitivity of the hydrogel at the body temperature is a desirable characteristic, which is likely to make easier the application of the formulations in the vaginal cavity. Once gelified, due to temperature increase, this hydrogel would develop mucoadhesive characteristics that are highly desirable for facilitating the presence of the NPs in the vaginal cavity and can help to maintain high local drug concentrations at the vaginal epithelium surface. Polymeric mucoadhesive hydrogels will be selected as a basis for creating an intimate and prolonged contact at the site of administration. An improved patient compliance is expected because of a possible reduction of the frequency of administration as well as the use of lower doses of drugs.

Obviously, conventional dosage forms such as solutions, ointments or creams cannot satisfy simultaneously these objectives, mostly because they are typically removed from the mucosal tissue within seconds to a few hours, thereby strongly limiting the duration of local formulations. Therefore, we propose in the present study a thermal analysis and *in vitro* studies of cell toxicity and trichomonacidal activity of drug-free hydrogel containing PIBCA NPs.

The first objective of this work was first to show the impact of NPs included into pluronic[®] F127 hydrogel on the micelles formations and its physico-chemical properties. Then the toxicity of the NPs was evaluated using histological study on vaginal pig mucosa and three different cell models: HeLa cells as non-oriented cells derived from the human cervical carcinoma, Caco2/TC7 and HT-29/MTX as differentiated and oriented cells from human colorectal adenocarcinoma characterized by the presence of the tight junctions, the HT-29/MTX cells have the particularity to produce mucus.

2. Materials

Pluronic[®] F127 (Poloxamer P407, EO₉₈PO₆₉EO₉₈) and pluronic[®] F68 (Poloxamer P188, EO₈₀PO₂₇EO₈₀) copolymers of pharmaceutical grade were purchased from BASF ChemTrade GmbH (Ludwigshafen, Germany). For cell culture: Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and fetal calf serum were from Invitrogen, Saint Aubin, France. Amino-Acid Non Essential 1 % (AANE 100X, Gibco, France). Trypsin and ethylenediamine tetraacetic acid EDTA were from Sigma-Aldrich (Saint-Quentin Fallavier, France), culture plates (TPP, Dutscher, France). Trypan blue was from VWR (France). Glucose and nonessential amino acids were from Life Technologies (Cergy Pontoise, France). All other reagents used for the preparation of simulated vaginal fluid (SVF) and phosphate buffer saline (PBS) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). SVF was prepared according to the protocol described in the supplementary data. Isobutylcyanoacrylate (IBCA) was a generous gift from Henkel Biomedical (Dublin, Ireland). Commercial chitosan (from Sigma-Aldrich, Saint-Quentin Fallavier, France) was depolymerized to reach the M_w of 20 000 g/mol) and thiolated as described in the supplementary data. The resulting polymer is chitosan-4-thiol-butylamidine named Chito20-TBA with a degree of deacetylation of 60 %.

Bactotryptone and Yeast extract were from BD Difco. Maltose, L-cysteine hydrochloride and L-ascorbic acid were from Sigma-Aldrich (Saint-Quentin Fallavier, France). All other reagents were supplied by Sigma-Aldrich (Saint-Quentin Fallavier, France) and were of analytical grade.

T. vaginalis strain CMP (Châtenay-Malabry Parasitology) was isolated from a woman suffering from STD in 1987 and stored as stabilized in liquid nitrogen containing 6 % dimethyl sulphoxide (DMSO) as cryoprotectant. This strain was metronidazole-sensitive.

3. Methods

3.1. Preparation of NPs

NPs were prepared by anionic emulsion polymerization according to the method of (Bravo-Osuna et al. 2006). Briefly, 0.069 g of mixtures of hydrolyzed and thiolated chitosan (Chito20/Chito20-TBA) at different percentages (25/75 %, 50/50 %, 75/25 % and 100/0 %) were dissolved in 5 mL of 0.2 M nitric acid, in a glass tube at 40 °C, under vigorous magnetic stirring and argon bubbling. After 10 min, 0.25 mL of IBCA were added. Argon bubbling was kept for an additional 10 min and stopped. The reaction was allowed to continue at 40 °C under vigorous magnetic stirring for 50 min or 24 h. The final formulation was stored at 4 °C until use. To simplify the notation NPs polymerized during 24 h will be noted (24 h).

Control PIBCA nanoparticles were prepared similarly without adding chitosan and stabilized with 1 % w/v pluronic[®] F68.

NP suspensions were purified by dialysis using a Spectra/Por[®] membrane with molecular weight cut off of 100 000 g/mol, twice during 30 min, then during 60 min and 90 min and once overnight against 1 L of acetic acid solution 16 μ M.

3.2. Physico-chemical characterization of the NPs

The mean hydrodynamic diameter of the NPs and their size distribution were determined from the Z-average obtained between by quasi-elastic light scattering using Zetasizer Nanoseries (Malvern Instruments Ltd. UK). The scattered angle was fixed at 173° and 60 μ L of each sample was diluted in 2 mL of acetic acid 0.16 μ M (Millex, SLAP 0225, Millipore, France). Zeta potential of NPs was measured using Zetasizer Nanoseries (Malvern Instruments Ltd. UK). The dilution of the suspensions (1:33 (v/v)) was performed in NaCl (1 mM).

The mean hydrodynamic diameter of the NPs was measured in the presence or not of pluronic[®] F127 at different concentrations between 0.5 to 20 wt%.

Each experiment was replicated three times.

3.3 Preparation of the hydrogels

Hydrogels were prepared according to the so-called “cold method” (Aka-Any-Grah et al. 2010). Pluronic[®] F127 hydrogel (20 wt%) was obtained by gradually adding under stirring the pluronic[®] F127 powder to water maintained at 4 °C. The hydrogel containing the NPs was prepared by slowly dispersing under magnetic stirring pluronic[®] F127 powder (20 wt%) directly in the NP dispersion at 4 °C until the complete dissolution of the polymer. The

concentration of the NPs into the hydrogel was changed as: 20 and 10 mg/mL.

3.4. Thermal analyses of the formulations

3.4.1. Rheology

Temperature ramp: Rheological measurements were performed on a controlled stress AR-G2 rheometer (TA Instrument Ltd., London, UK) using a cone-plate geometry (diameter 60 mm, angle 2° and gap 55 µm). A solvent trap maintained a solvent-saturated atmosphere around the cell and evaporation was not significant for the investigated temperatures and the time scales.

Elastic (or storage) (G') and the viscous (or loss) moduli (G'') were recorded under a constant strain of 0.5 % to be in the linear viscoelastic regime in which the moduli are independent of the strain, at a frequency of 1 Hz. The presence of Pelletier device, placed in the lower plate, allowed temperature sweeps from 5 to 50 °C to be performed with a precision of 0.1 °C. The temperature at which G' suddenly increase corresponds to the sol-gel transition temperature.

After the rheology measurement conducted on the formulations, IBCA NPs were added to pluronic[®] F127 hydrogel to compare their rheological properties.

3.4.2 Micro-DSC

Micro-DSC experiments were carried out with a calorimeter Micro-DSC III Evo Setaram (Caluire, France) in the aim to determine the critical micelle temperature (CMT) (Pham

Trong, Djabourov, and Ponton 2008; Aka-Any-Grah et al. 2010; Petit et al. 2012). The cells used for the sample and the reference (distilled water or buffer) were the batch type (1 mL). Two empty cells with the caps were weighed after complete drying and the seals were chosen to obtain the same mass of the empty cells (± 0.2 mg). The sample and the reference were introduced into the cells at room temperature and weighed for the identical mass (± 0.3 mg). After their insertion into the oven at room temperature, the temperature of the oven was lowered to 5 °C with a rate of -0.2 °C/min, and then it were kept at this constant temperature to equilibrate the thermal flow during 1 hour. The measurement was performed at a heating rate of +0.1 °C/min until 50 °C. Afterwards, the oven was maintained at this temperature for 1 h. Then, it was started the melting with a rate of -0.1 °C/min to 5 °C. The analysis of the heat flows led to the determination of the CMT according to the previously described and well-known method (Pham Trong, Djabourov, and Ponton 2008).

3.5. Cell lines and culture conditions to evaluate the toxicity of the formulations

3.5.1. Culture of HeLa cells

Human cervix HeLa cells were grown in DMEM with L-glutamine (Invitrogen, Fisher, France) supplemented with 10 % heat-inactivated fetal calf serum (Invitrogen, Fisher, France) and 1 % Amino-Acid Non-Essential (AANE 100X, Gibco, Invitrogen, Fisher, France) at 37 °C in an atmosphere containing 5 % CO₂. Cell lines were harvested from the flask using trypsin (0.5 mg/mL) or EDTA (0.2 mg/mL), washed once with culture medium, and seeded into culture plates (TPP, Dutscher, France) at the appropriate cell densities

(150,000 cell/well), before being incubated at 37 °C in an atmosphere containing 5 % CO₂ before experiments began.

For maintenance purposes, cells were passaged weekly using 0.02 % trypsin in Ca²⁺ Mg²⁺ - free PBS containing 3 mM EDTA. Experiments and cell maintenance were carried out at 37 °C in a 10 % CO₂/90 % air atmosphere. The culture medium was changed daily.

The evaluation of the toxicity of the formulations was achieved by exposing HeLa cells to pluronic[®] F127 hydrogel (20 wt%) containing 10 or 20 mg/mL of PIBCA NPs during 24 h. The results were compared to formulations without NPs (pluronic[®] F127 20 wt%) and to a solution of NPs in an aqueous solution at pH 4.5. This solution was prepared by adding NaOH 0.2 N drop by drop to a solution of HNO₃ 0.2 N until pH 4.5. This protocol was used to mimic the polymerization medium.

HeLa cells were seeded in a 24-well culture plate at 1.5×10^5 cells and incubated for 24 h at 37 °C in 5 % CO₂ prior to the test. Prior to contact, the confluent HeLa cells were washed twice with PBS, and then put in contact with hydrogels at appropriate concentration. The plates were incubated for 24 h at 37 °C in a humidified atmosphere containing 5 % CO₂. At the end of the contact period, the cells were washed two times with sterile PBS to remove non-adhering gels and then processed for analysis.

The quantification of viable HeLa cells was conducted with Trypan blue numeration (VWR, France) in Malassez cell.

For statistical analysis t student's t-test was applied ($p = 0.05$).

3.5.2. Culture of Caco-2/TC7 clone cells

The enterocyte-like Caco-2/TC7 clone cells (Chantret et al. 1994) established from the parental Caco-2 cells (Fogh et al. 1977) were routinely grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) with 25 mM glucose, supplemented with 15 % heat-inactivated (30 min at 56 °C) fetal calf serum and 1 % nonessential amino acids. For maintenance purposes, the cell lines were passaged weekly, using 0.02 % trypsin in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) containing 3 mM EDTA. Monolayers were prepared in 24-well tissue culture plates (Iwaki Glass) by seeding 7×10^4 cells per well. Experiments and cell maintenance were carried out at 37 °C in a 5 % CO_2 –95 % air atmosphere. The culture medium was changed daily. Assays were performed with cells at passages between 49 and 55 for Caco-2 cells. For maintenance purposes, cells were passaged weekly using 0.02 % trypsin in Ca^{2+} Mg^{2+} free PBS containing 3 mM EDTA. Experiments and cell maintenance were carried out at 37 °C in an atmosphere of 10 % CO_2 –90 % air. The culture medium was changed daily. Fully differentiated cells were obtained after 15 days in culture (Moal and Servin 2013).

3.5.3 Culture of HT-29/MTX cells

We used the mucin-secreting HT-29/MTX cell subpopulation (Lesuffleur et al. 1990) established from the cultured human colonic adenocarcinoma parental HT-29 cell line (Fogh et al. 1977). Cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (25 mM glucose), supplemented with 10 % heat-inactivated (30 min, 56 °C) fetal bovine serum (FBS) (Invitrogen). The enterocyte-like HT-29 $\text{glc}^{-/+}$ cell subpopulation (Lesuffleur et al. 1991) has been adapted for culture in the presence of glucose from the enterocyte-like HT-29 glc^- cell subpopulation (Zweibaum et al. 1985) established

from the cultured human colonic adenocarcinoma parental HT-29 cell line (Fogh et al. 1977). Cells were routinely grown in DMEM supplemented with 20 % heat-inactivated FBS. For maintenance purposes, cells were passaged weekly using 0.02 % trypsin in Ca^{2+} Mg^{2+} free PBS containing 3 mM EDTA. Experiments and cell maintenance were carried out at 37 °C in an atmosphere of 10 % CO_2 –90 % air. The culture medium was changed daily. Cultures were used at late postconfluence after 21 days in culture (Moal and Servin 2013).

3.6. Evaluation of the toxicity of the formulations on vagina pig mucosa

Experiments were carried out on female pigs (INRA Jouy en Josas, France) weighing between 60 and 63 kg in average. The animals were fasted for 24 h but had free access to tap water. All experiments on animals adhered to the Communities Commission Directive (DE/86/609/CEE) and were performed in conformance with the French Ministry of Agriculture Permission No. 78-16. Pigs were sacrificed by intravenous injection (20 mL) of overdosed sodium phenobarbital (Dolethal, Vetoquinol Laboratory, Lure, France), and the vaginal mucosa was taken over a 10 cm length. The mucosa was placed in SVF and stored at -20 °C immediately after sacrifice of the animal and kept at this temperature until use. It has been shown that porcine mucosa can be frozen during storage without affecting the mucus layer (Squier et al. 2008). Samples were cut in 1 cm square pieces by means of surgical scissors to obtain intact vaginal tissue and defrosted before experiments at ambient temperature in the presence of SVF.

Mucosa was placed in Franz cell and the receptor compartment was filled with SVF at pH 4.5.

Then, SVF, pluronic[®] F127 20 wt%, NPs PIBCA/(Chito20/Chito20-TBA) (75/25 %) and NPs Chito20/Chito20-TBA (75/25 %) gelified with pluronic[®] F127 20 wt% were placed on the mucosa and Franz cell were closed and placed in a thermostated bath at 37 °C.

After prior fixation in FineFix, (Milestone, Italy), embedding in paraffin, deparaffinized 4 µm thick slides were stained with hematoxylin, eosin and saffranin. The light micrographs of specimens are obtained with a Microscope Axiophot Zeiss (Germany) connected to a digital camera (PCO, Germany). Whole specimens were scanned with a slide scanner (Nikon Super Coolscan 8000) customized for histopathology (Groupe Régional d'études sur le cancer, Caen, France).

3.7. Evaluation of the anti-*T. vaginalis* activity of the formulations

T. vaginalis was cultured axenically at 35 °C in trypticase-yeast extract-maltose (TYM) (Diamond 1957) supplemented with 10 % horse serum (Gibco, France) and subcultured every 2 days (Camuzat-Dedenis et al. 2001).

Culture tubes with fresh TYM medium enriched with filtered horse serum 10 % alone or with the tested formulations, were inoculated with 2×10^5 protozoa in 3 mL. The tubes were cultivated in batch conditions for 24 h at 35 °C and the number of parasites per milliliter in each tube determined with an haemocytometer (Kova[®] Glasstic[®] Slide 10, Hycor Biomedical, United States). The experiments were run three times.

The results were estimated as the percentage of growth inhibition compared with only excipient treated controls and plotted as profit value as a function of drug concentration (n = 3).

For statistical analysis, a t student's t-test was applied ($p = 0.05$).

4. Results and discussion

The aim of the present work was to investigate the possibility to enhance the microbicidal activity of vaginally-administrated formulations by using hydrogels containing PIBCA NPs. Before adding any drug to the formulation, it was interesting to evaluate the specific trichomonacidal potential of this formulation, considering the antiprotozoal efficacy of PIBCA against African trypanosomes (Lherm et al. 1987). Hydrogels and NPs were first characterized from a physico-chemical point of view and thermal analysis. The toxicity against different cells models and tissue and the efficacy against *T. vaginalis* were then studied.

4.1. Nanoparticle characterization

The formulation developed here is composed of NPs composed of PIBCA synthesized by anionic emulsion polymerization (Figure 1). These NPs are composed of a hydrophobic core of PIBCA and are coated with different percentages of chitosan and thiolated chitosan or with pluronic® F68. The NP size was in the range of 185-210 nm, in agreement with previous data (Mazzaferro et al., 2011). The polymerization duration did not have significant impact on the NP size. From the electrophoretic mobility measurements, it was deduced that chitosan-coated PIBCA nanoparticle zeta potential was positive. The values of zeta potential varied from +34.3 to +40.4 mV. This indicated that the cationic chitosan was located at the surface of the nanoparticles and completely masked the negative surface charge found on control PIBCA nanoparticles stabilized with pluronic® F68 (-7.5 mV) in agreement with previous works

(Petit et al., 2012).

Table 1. The mean hydrodynamic diameter (D) and ζ potential of PIBCA/(Chito20/Chito20-TBA) NPs prepared by anionic emulsion polymerization. Control is composed of PIBCA nanoparticles not coated with chitosan stabilized with pluronic[®] F68. The polymerization duration was kept 1 h or 24 h. (n=3).

Polymerization duration (h)	Chito20/Chito20-TBA (%)	D (nm)	ζ potential (mV)
1	100/0	185.4 ± 2.5	$+39.6 \pm 0.6$
	75/25	210.6 ± 3.8	$+34.3 \pm 1.0$
	50/50	196.0 ± 1.6	$+39.0 \pm 0.4$
	25/75	192.3 ± 1.8	$+40.4 \pm 0.8$
24	75/25	215.8 ± 1.4	$+39.5 \pm 0.7$
1	Control	189.4 ± 2.5	-7.5 ± 0.3

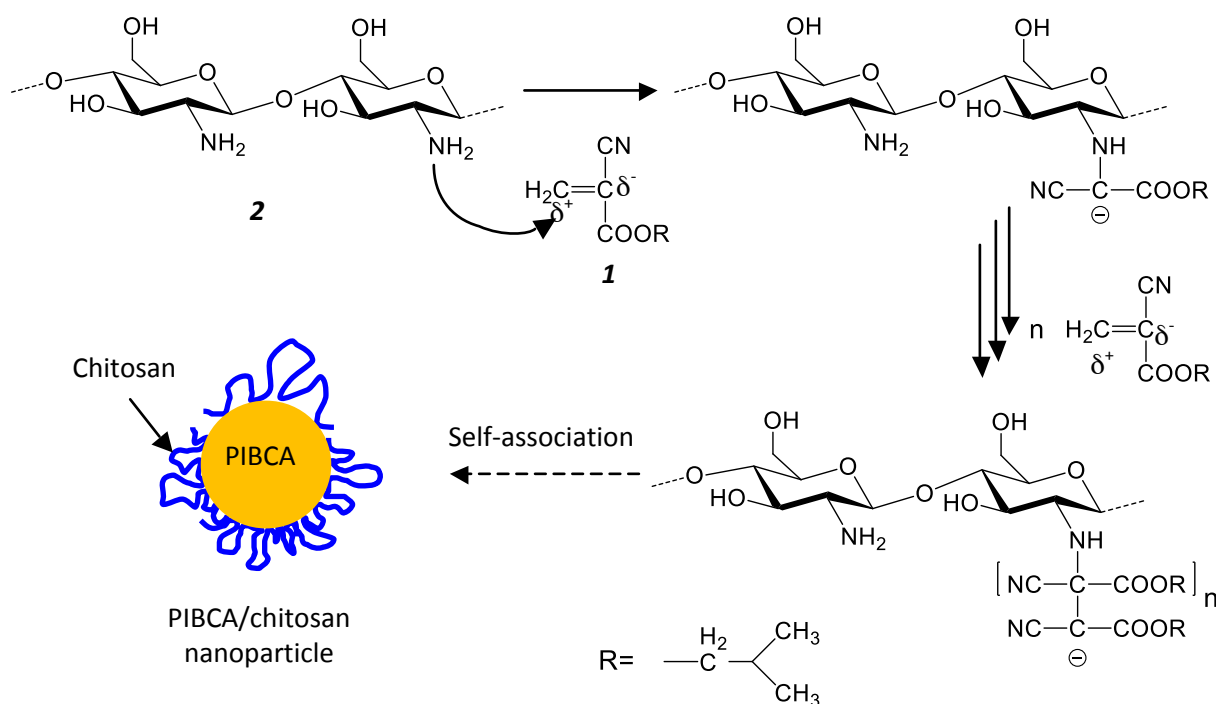


Figure 1: Schematic representation of the initiation of the polymerization of isobutylcyanoacrylate (**1**) by the deacetylated units of chitosan (**2**) according to anionic mechanism. PIBCA: poly(isobutylcyanoacrylate).

4.2. Effect of NP addition on rheological properties of the formulations

The aim of this part of the work is to investigate the effect of the pH and the addition of the NPs Chito20/Chito20-TBA (75/25 %) on pluronic[®] F127 hydrogel properties. Particularly, variations in sol-gel transition temperatures were analysed.

Usually, the temperature of vaginal hydrogels is considered to be suitable for vaginal administration when they are lower than the human vaginal temperature (37.2 °C) (Rashad et al. 1992). If gelling temperature is higher than 37.2 °C, hydrogels will lose their gelling properties resulting in a leakage of the formulations from the vagina (Ibrahim, 2012).

Initially, the thermosensitive properties of the hydrogels were evaluated considering their sol/gel transition temperature. The shear moduli G' and G'' from different preparations are presented in Figure 2. Particularly, formulations in the absence (Fig. 2A and 2B) and in the presence (Fig. 2C and 2D) of NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %) are shown. the storage modulus G' , is very low in solution state and increases drastically when gelling temperature is reached. (Wei et al. 2002). As can be seen from the results reported in Table 2, gelling temperatures were in all cases lower than body temperature and showed low variations in the presence of NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %).

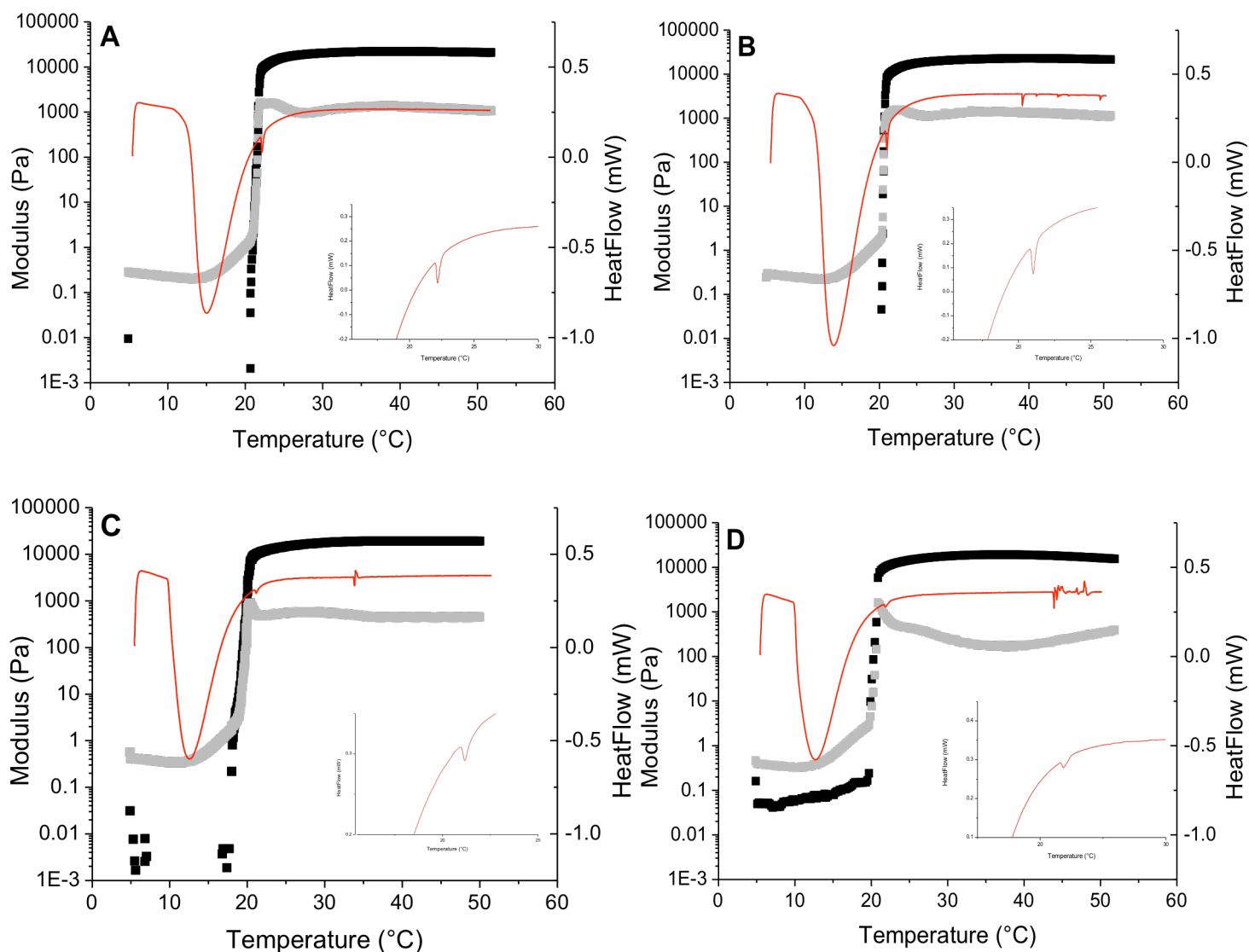


Figure 2: Variation of storage modulus G' (black symbols) and loss modulus G'' (grey symbols) versus temperature and heat flow (plain line) trace for (A) F127 20 wt% in water, (B) F127 20 wt% at pH 4.5, (C) F127 20 wt% at pH 4.5 containing 20 mg/mL of NPs PIBCA/(Chito20/Chito20-TBA) (75/25%) and (D) F127 20 wt% at pH 4.5 containing 10 mg/mL of NPs PIBCA/(Chito20/Chito20-TBA) (75/25 %).

Table 2. Rheological characterization of empty or NPs-containing formulations at pH 7 or 4.5 T_{gel} (gelling temperature), T_c (crystallization temperature), G' and G'' data were extrapolated from Fig. 2.

Formulation	T_{gel} (°C)	G' (10^3 Pa)	G'' (10^3 Pa)	T_c (°C)
F127 20 wt% Water	22.1 ± 0.1	20.3 ± 0.2	10.4 ± 0.1	22.1 ± 0.1
F127 20 wt% pH 4.5	20.8 ± 0.1	20.9 ± 0.3	11.3 ± 0.2	20.9 ± 0.1
F127 20 wt% + NP ^a 10 mg/mL	20.6 ± 0.1	18.4 ± 0.1	4.5 ± 0.3	21.7 ± 0.1
F127 20 wt% + NP ^a 20 mg/mL	20.2 ± 0.1	17.1 ± 0.2	7.8 ± 0.2	21.2 ± 0.1

^a NPs Chito20/Chito20-TBA (75/25 %) polymerization 1 h.

In order to establish the relation between the micelle formation and changes of rheological properties, we need to know: (i) if the structure is in equilibrium with temperature (heating and cooling ramps), (ii) if the copolymer is completely assembled in micelles at the end of the heating ramp. Microcalorimetric measurements were used to clarify these points. Heat flow curves during heating and cooling ramps at a rate of 0.1 °C/min are shown in Fig. 3 and 4 for different samples. In the absence of NPs in the pluronic[®] F127 hydrogel, there was a perfect symmetry (mirror image) of the heat flow measured during cooling and heating. This was observed whatever the concentration of pluronic[®] F127. This showed that there was no hysteresis between these thermal histories: indicating that the micelle solution was in equilibrium at each temperature and its local structure changed reversibly (Fig 3A, 3B and 4.). However, after the addition of NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %) the heat flows during heating and cooling were modified. The hydrogel containing NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %) was not totally reversible (Fig 3C, 3D and 4.).

The endothermic micellization process can be analyzed as usual by the determination of the onset temperature (T_{onset}), the peak temperature (T_{peak}) and the endset temperature (T_{endset}) (Fig 3A) and by the calculation of the area under the main peak corresponding to the enthalpy of transition. The onset is the temperature where micelles start to form and the endset is the temperature where the micellization was completed. In between, at each intermediate temperature, the area under the peak calculated from this onset temperature increases proportionally with the amount of formed micelles.

Experiments were repeated for different concentrations of nanoparticles and pluronic[®] F127. The results are shown in Figures 2, 3 and 4.

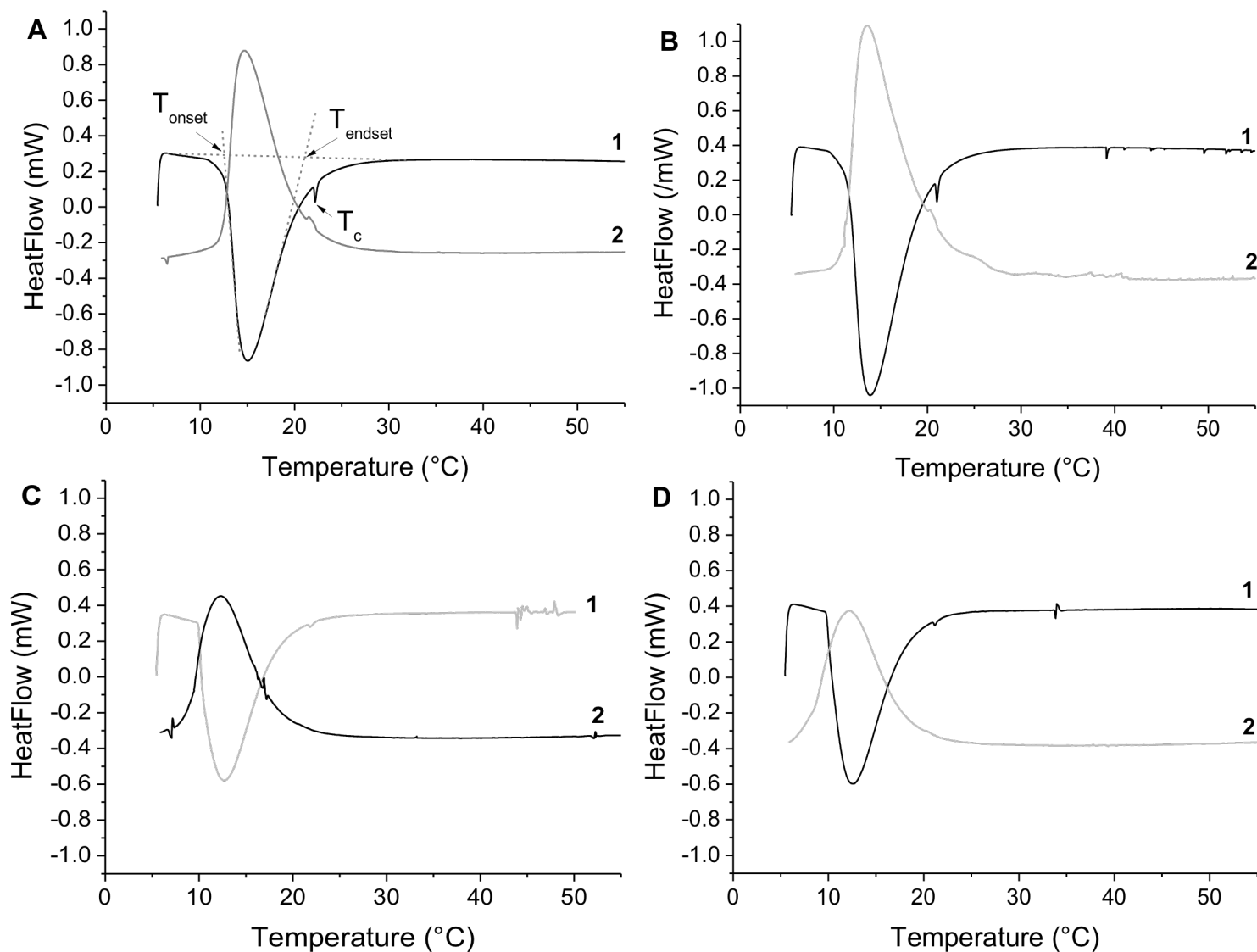


Figure 3. Micro-DSC curves showing the micellization and crystallization peaks during heating (1) and cooling (2) for: **(A)** pluronic® F127 20 wt% at pH 7 and definition of T_{onset} , T_{peak} , T_{endset} and T_c ; **(B)** pluronic® F127 20 wt% at pH 4.5; **(C)** 10 mg/mL of NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %) mixed with pluronic® F127 20 wt%; **(D)** 20 mg/mL of NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %) mixed with pluronic® F127 20 wt%.

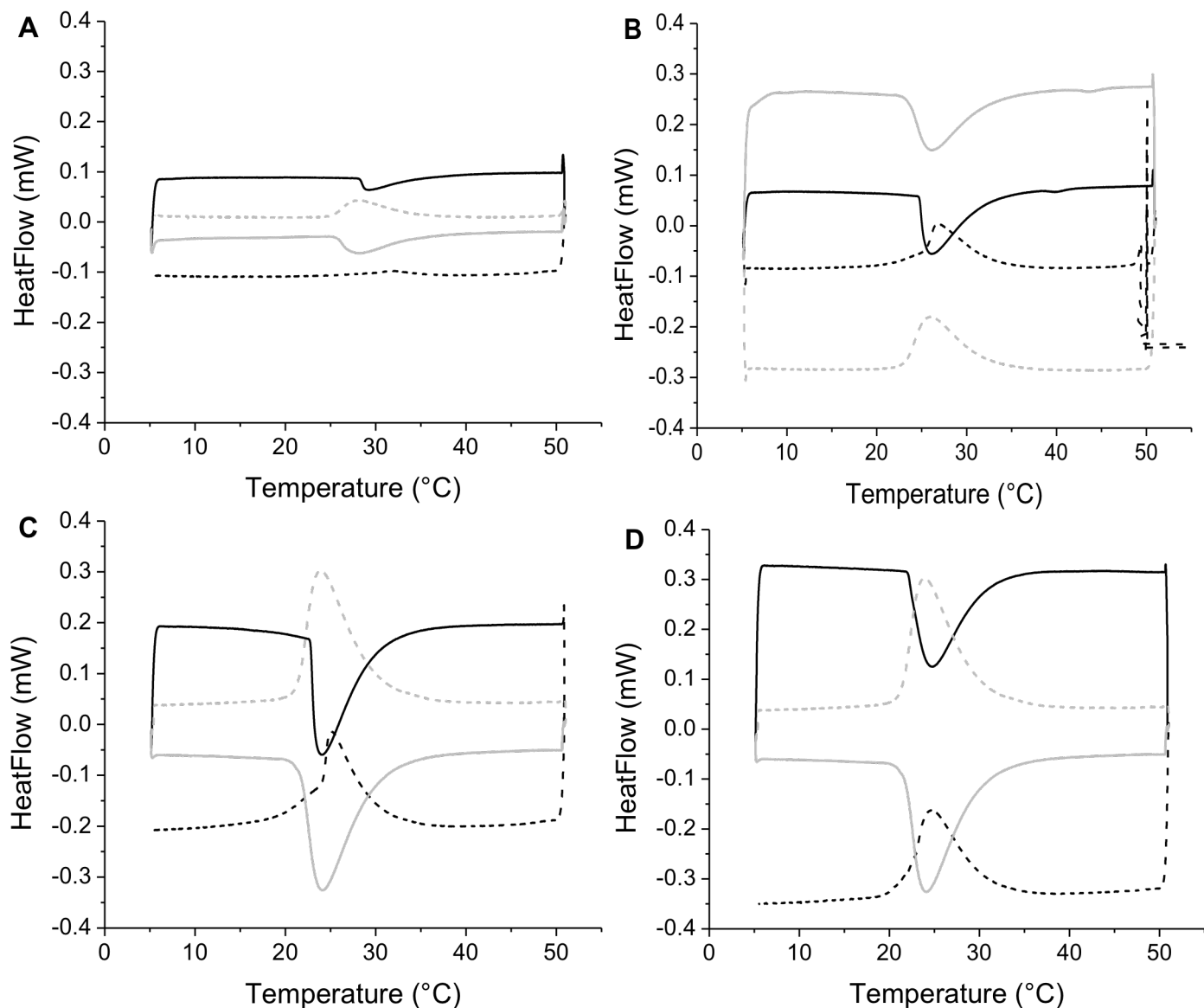


Figure 4. Micro-DSC heat flows showing the micellization and crystallization peaks during heating (solid line) and micelle melting during cooling (dash line) for: **(A)** pluronic® F127 at 0.6 wt% at pH 4.5 with (black line) or without (grey line) 10 mg of NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %); **(B)** pluronic® F127 at 2 wt% at pH 4.5 with (black line) or without (grey line) 10 mg of NPs PIBCA/(Chito20/Chito20-TBA) (75/25 %); **(C)** pluronic® F127 at 4 wt% at pH 4.5 with (black line) or without (grey line) 10 mg of NPs PIBCA/(Chito20/Chito20-TBA) (75/25%); **(D)** pluronic® F127 at 4 wt% at pH 4.5 with (black line) or without (grey line) 10 mg of NPs PIBCA/(Chito20/Chito20-TBA) (75/25 %) polymerization 24 h.

These experiments (Figure 2) demonstrated that the onset temperature also was lowered from 12.1 to 10.9 °C when the pH of the formulations varies from 7.0 to 4.5. (Figures 2A, 2B and Table 3). This result may be explained by the solvation effect due to the presence of ions in the formulations at pH 4.5. However the T_{gel} was still lower than the body temperature suggesting that the formulations should be able to remain in the gel state after their *in vivo* administration. The enthalpy of micellization was larger than the other at pH=4.5 in the absence of NPs. The enthalpy per gram is constant in the micellization of pluronic® F127 (Table 3).

The onset and gelling temperatures were almost identical for gel composed of 20 wt% of pluronic® F127 in water or at pH 4.5 (Figures 2A and 2B) but they were shifted after the addition of NPs Chito20/Chito20-TBA (75/25 %) (Figures 2C and 2D).

At the molecular level, we can postulate that the quantity of pluronic® F127 engaged in the micelles was changed in presence of NPs. If enthalpy of formation is superior of enthalpy of melting, during fusion only a portion of pluronic® F127 was redispersed.

The sol gel transition (when the shear modulus dramatically increases) was directly related to the crystallization peak of the micelles formed between the onset and the endset temperatures. The crystallization peak was apparently smaller when the NPs are included in the hydrogel suggesting that they are able to hinder or delay the crystal formation. In Table 2 we can see that the shear moduli in presence of NPs in the gel state were smaller than in the pure pluronic® hydrogel at the same concentration. The presence of NPs into pluronic® F127 hydrogel results in lower moduli, which still remain very high due to the crystalline structure of the hydrogel.

In the presence of NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %), micro-DSC experiments revealed that the reversibility of the hydrogel was lost (Figure 3). With a low concentration of pluronic[®] F127, the phenomenon was amplified and could be better observed. In a solution containing 10 mg/mL of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs with lower concentrations of pluronic[®] F127 (0.6 wt%, 2 wt% or 4 wt%) are shown Figures 3A, 3B and 3C. In the absence of NPs, the heat flows measured during cooling and heating are perfectly symmetric and the areas under the peaks were equal (Table3). In the presence of NPs, the peak area during heating was larger than during cooling (Table 3).

The micellization peak was smaller in the presence of NPs indicated that not all pluronic[®] F127 unimers participate to micelle formation. Part of them interact with the NPs, they may be attracted at the particle surface. According to the area of the micellization peaks, the decrease of enthalpy is of the order of 30 % (from 30 J/g to 20 J/g) for NPs Chito20/Chito20-TBA (75/25 %) 10 mg/mL in presence of pluronic[®] F127 20 wt% and as large as 40 % (from 30 J/g to 12 J/g) for PIBCA/(Chito20/Chito20-TBA) (75/25 %) 10 mg/mL in the presence of pluronic[®] F127 0.6 wt%. When solutions were cooled again allowing the micelles to be redispersed (melted) it is observed that in the presence of the NPs, micelles were more stable to the temperature, the melting peaks were more spread towards high temperatures. One can suppose that some of the micelles contain complexes between unimers and poorly soluble molecules. Such molecules should originate from the NPs themselves, which upon heating could release short PIBCA polymers. Therefore we decided to examine the behavior of NPs in diluted solutions by DLS, looking for changes of the particle diameters.

Figure 5 shows the diameters of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs in solutions containing various pluronic[®] F127 concentrations: it was observed that the size increases with F127 concentration. For instance an additional of 4 % pluronic[®] F127 the size

increased by 100 nm, from 200 nm to 300 nm. Compared with the original size in the absence of pluronic[®] F127 this experiment is consistent with our hypothesis that unimers of pluronic[®] F127 may be adsorbed on the NPs Chito20/Chito20-TBA (75/25 %) and that they don't contribute the micellization of pluronic[®] F127 hence modifying the gelation.

Table 3. T_{onset}, T_{peak}, T_{endset} and T_c, of pluronic[®] F127 with and without NPs, micellization peaks deduced from Micro-DSC experiments.

Formulation	Process	T _{onset} (CMT) (°C)	T _{peak} (°C)	T _{endset} (°C)	T _c (°C)	ΔH (J/g) ^c	$-\frac{\Delta H_{Cooling}}{\Delta H_{Heating}}$
F127 20 wt% pH 7	Heating	12.1	15.3	21.3	22.1	-23.79	1.06
	Cooling	11.9	14.7	21.3	21.6	25.26	
F127 20 wt% pH 4.5	Heating	10.9	13.9	20.1	20.9	-30.08	1.13
	Cooling	10.8	13.5	20.3	20.3	33.86	
F127 20 wt% + NP ^a 10 mg/mL	Heating	9.8	12.7	18.5	21.7	-20.15	0.98
	Cooling	8.1	12.2	18.1	20.9	19.81	
F127 20 wt% + NP ^a 20 mg/mL	Heating	9.7	12.5	19.1	21.2	-19.52	0.92
	Cooling	6.9	12.4	17.8	20.3	17.95	
F127 4 wt% pH 4.5	Heating	21.6	24.1	30.3	- ^d	-26.06	1.02
	Cooling	21.1	23.9	30.1	- ^d	26.47	
F127 4 wt% + NP ^a 10 mg/mL	Heating	22.6	23.9	30.2	- ^d	-20.38	0.96
	Cooling	17.5	25.0	30.5	- ^d	19.50	
F127 2 wt% pH 4.5	Heating	22.8	26.3	32.5	- ^d	-28.75	0.93
	Cooling	23.1	25.9	32.5	- ^d	26.88	
F127 2 wt% + NP ^a 10 mg/mL	Heating	24.5	26.3	32.7	- ^d	-25.69	0.71
	Cooling	20.7	26.7	33.8	- ^d	18.25	
F127 0.6 wt% pH 4.5	Heating	25.2	28.2	34.9	- ^d	-28.13	0.95
	Cooling	25.1	28.2	34.8	- ^d	26.67	
F127 0.6 wt% + NP ^a 10 mg/mL	Heating	27.8	29.2	34.9	- ^d	-12.29	0.64
	Cooling	25.9	31.9	35.8	- ^d	7.92	
F127 4 wt% + NP ^b 10 mg/mL	Heating	21.9	24.8	30.4	- ^d	-20.97	0.94
	Cooling	21.4	24.7	30.6	- ^d	19.78	

^a NPs Chito20/Chito20-TBA (75/25 %) polymerization 1 h

^b NPs Chito20/Chito20-TBA (75/25 %) polymerization 24 h

^c Enthalpy per g of pluronic[®] F127

^d No crystallization

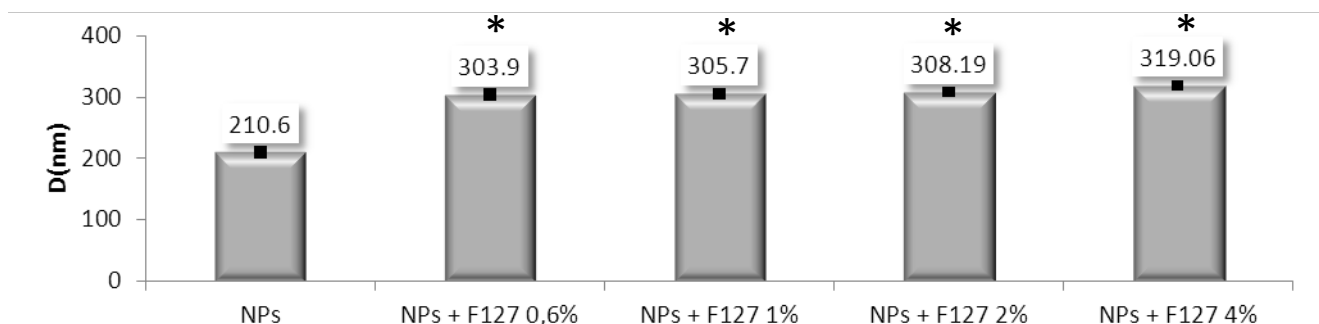


Figure 5: Mean hydrodynamic diameters of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs in the presence of different concentrations of pluronic® F127 at 20 °C. *Student test at 0.05 %.

The size of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs was measured at different temperatures in the presence of 0.6 % and 4 % of pluronic® F127 (Fig 6A. and 6B.). It is clearly seen that the size of NPs decreases during heating from 319 nm to 160 nm for example in presence of pluronic® F127 4 wt% (Fig 6B.); and increases back during cooling. However, the NPs don't recover their initial size after heating. The size changes depend of the pluronic® F127 concentration and are initiated at the close to the onset temperature of the micelle formation. For instance, in the presence of 0.6 wt% of pluronic® F127, the NP size decrease begins at 35 °C, and with 4 wt% of pluronic® F127 at 25 °C (Fig 6A. and 6B.). The decrease is progressive with temperature.

These experiments show that NPs were not inert versus temperature changes and they are able to partly release some components into the aqueous solution. The polymer PIBCA being essentially hydrophobic could migrate inside the micelles at high temperature and help to stabilize their structure. The non-reversibility between micelle formation and melting might be explained by the incorporation of these hydrophobic molecules (PIBCA) in the micelles.

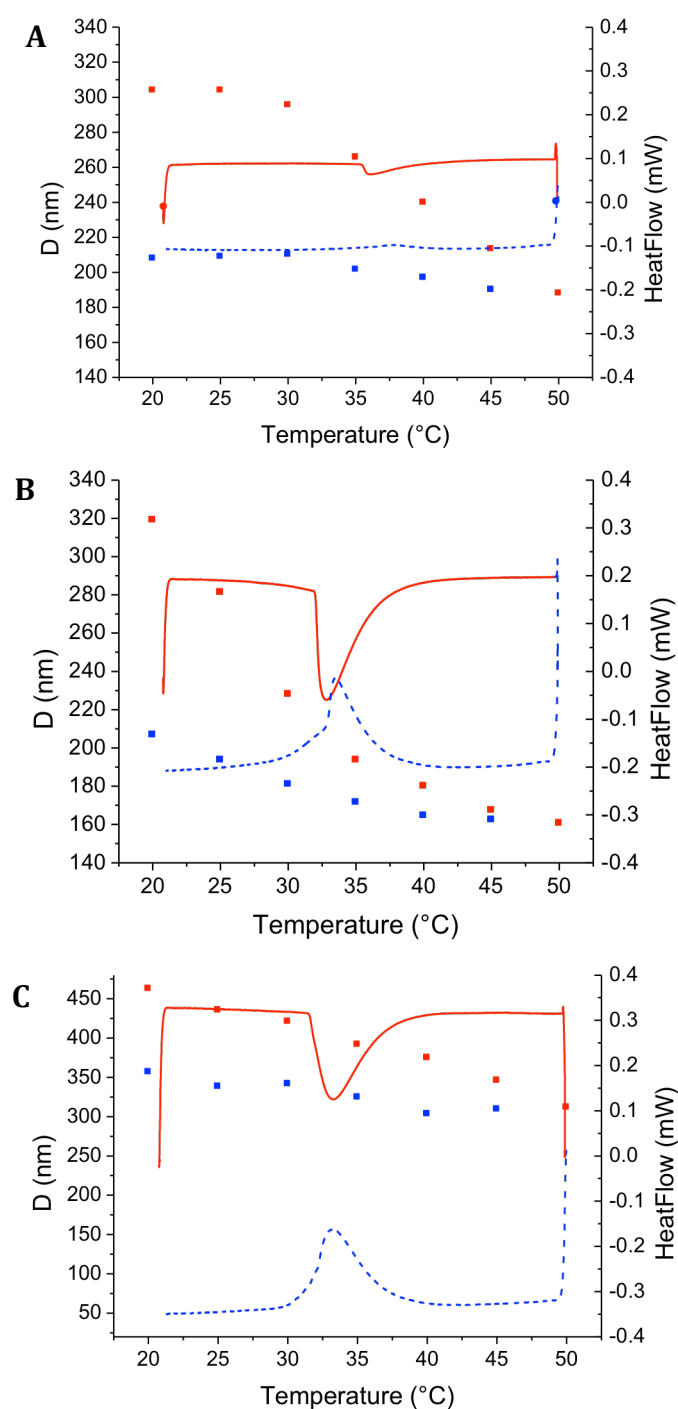


Figure 6: Size of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs during heating (■) and cooling (■) and Micro-DSC measurements during heating (red line) and cooling (blue dot line) in the presence of: **(A)** F127 0.6 wt% at pH 4.5 with 10 mg of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs; **(B)** F127 4 wt% at pH 4.5 with 10 mg of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs; **(C)** F127 4 wt% at pH 4.5 with 10 mg of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs polymerization 24 h.

The study was completed by two other complementary experiments to elucidate the question about the stability of the NPs. First, the polymerization time of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs was extended to 24 h instead of 1 h and dialyses were performed. It has been shown that NPs polymerized during 24 h were more stable than NPs polymerized during only 1 h. After 1 h of dialysis the PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs we can observe that the size of NPs decreased from 210 nm to 130 nm (Figure 7A.). The size of NPs was however stable with temperature, but osmotic pressure was sufficient to destabilize PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs, contrary to PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs polymerized during 24 h that retained their original size (Figure 7B). So after only 1 h the polymerization was not totally finished and the PIBCA needed more time more time to formed stables NPs.

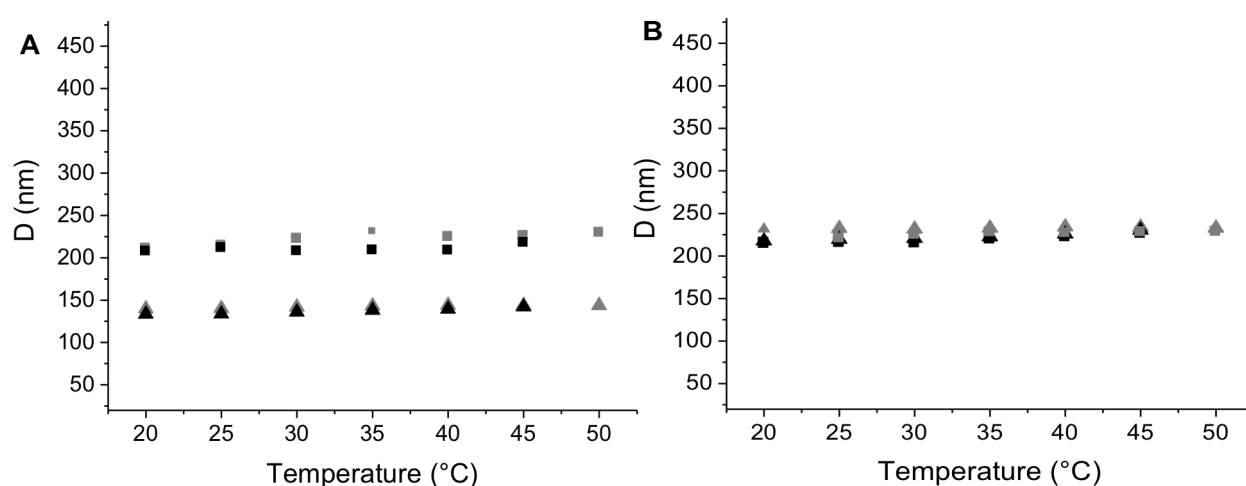


Figure 7: Size of PIBCA/(Chito20/Chito20-TBA) (75/25 %) NPs during heating (gray) and cooling (black) after dialysis (▲) or no (■) after (A) 1 h of polymerization and (B) 24 h of polymerization.

4.3. Evaluation of the cytotoxicity of the formulations on different cell lines

The cytotoxicity of the formulations was studied on three different cell line models after 24 h of contact with the formulations composed of the NPs of PIBCA/(Chit20/Chit20-TBA) and also pluronic[®] F127 20 wt% hydrogel. First, the cytotoxicity of the formulations was studied against HeLa cells, which are a human cervical carcinoma cell line. The results on Figure 8 indicated that all the HeLa cells after 24 h of incubation with NPs PIBCA/(Chito20/Chito20-TBA) (75/25 %) at a concentration of 20 mg/mL were dead. So, the NPs at a concentration of 20 mg/mL were very cytotoxic against these cells. This cytotoxicity was not due to the pH of the formulation (pH 4.5) and to the pluronic[®] F127 hydrogels. Indeed, without PIBCA NPs, a solution at pH 4.5 and the pluronic[®] F127 hydrogel have no significant cytotoxicity against HeLa cells (Figure 8).

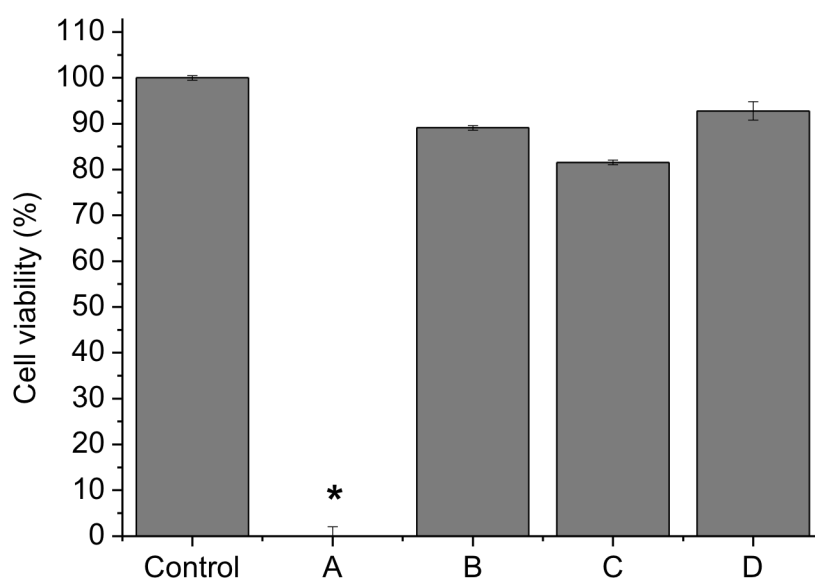


Figure 8. HeLa cell viability after 24 h of contact with NPs composed of PIBCA/(Chit20/Chit20-TBA) (75/25 %) at a concentration of 20 mg/mL (A), an aqueous solution at pH 4.5 (B), a hydrogel of pluronic[®] F127 20 wt% at pH 7 (C) and pH 4.5 (D). The results were compared to solution at pH 4.5. Culture medium (Control) was used as negative control. Data are the mean of three determinations \pm sd. * significant versus control $p < 0.05$. Student test at 0.05 %.

To complete the study, different concentrations of NPs with a shell containing different percentages of thiolated chitosan were incubated in contact with the human cervix HeLa cells. The results on Figure 9 showed that the NPs have high cytotoxicity against HeLa cells even at low concentrations and the thiol content did not seem to modify the HeLa cell viability.

To complete this *in vitro* study, the cytotoxicity of NPs against human polarized epithelial cells was evaluated using a model of enterocytes, the Caco-2/TC7 clone cells, and a model of mucus-secreting cells, the HT-29/MTX cell subpopulation (Figure 9). Whatever the thiol content in the NPs shell was, no significant cytotoxicity of the NPs was observed at a concentration of 12.5 or 25 $\mu\text{g/mL}$ against both Caco-2/TC7 and HT-29/MTX cells. In contrast, the NPs had a very strong toxicity against HeLa cells at these concentrations. This difference in toxicity could be explained by the fact that HeLa cells are undifferentiated unlike that the Caco-2/TC7 and the HT-29/MTX cells. It would be expected that the HT-29/MTX cells were more protected because they produce mucus that cover the apical cell surface and exert a physical barrier against the membrane passage of the formulation.

Accordingly, whatever the thiol content the NPs were strongly cytotoxic at concentrations of 12.5 or 25 $\mu\text{g/mL}$ in HeLa cells but not on Caco-2/TC7 and HT-29/MTX cells. So, these NPs could be considered to be used to deliver drugs on a healthy colorectal epithelium but they are inappropriate to be used on a damaged epithelium which includes undifferentiated cells due to desquamation.

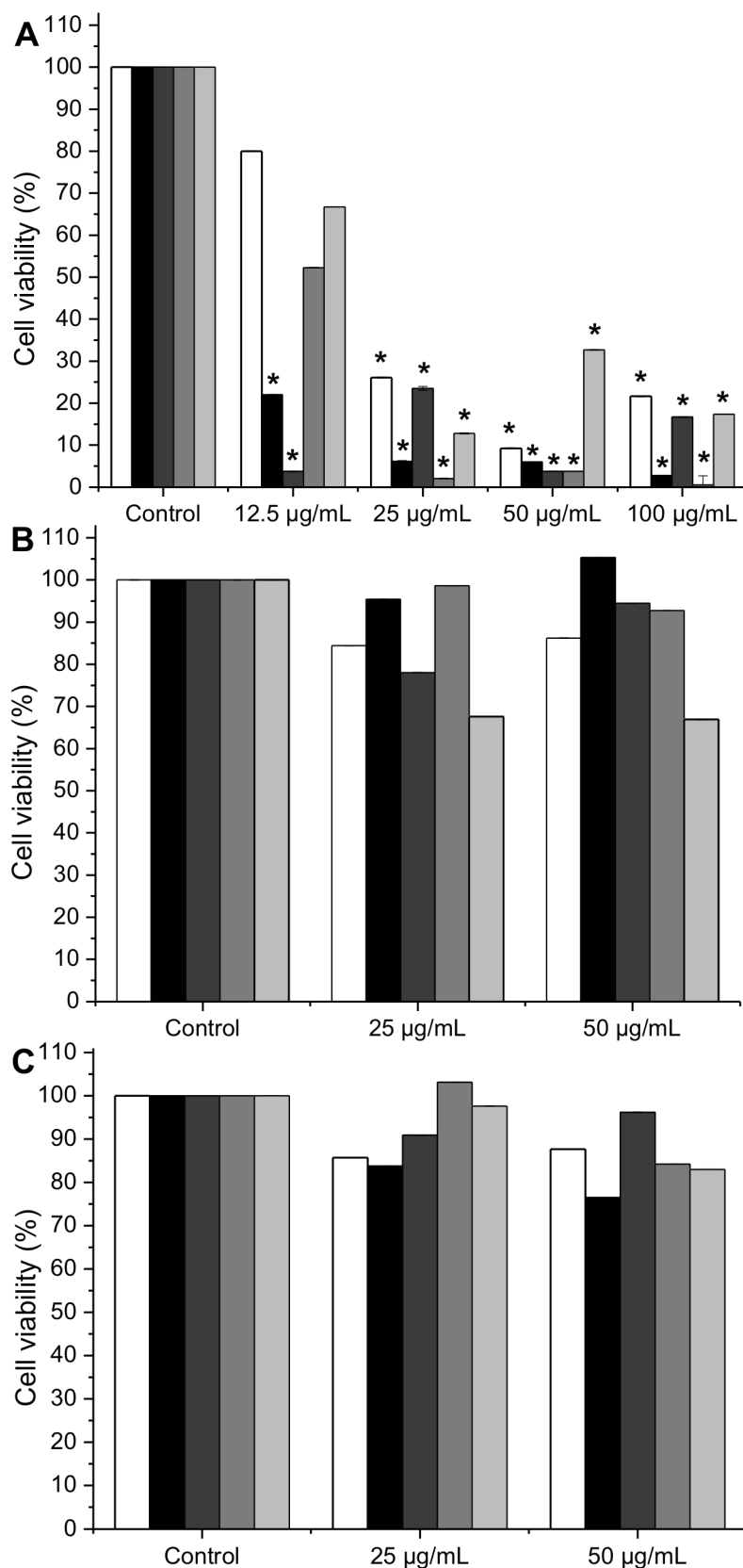


Figure 9. Cell viability of HeLa (A), Caco-2/TC7 (B) and HT-29/MTX (C) after 24 h of contact with different concentrations of PIBCA NPs coated with different percentages of Chito20/Chito20-TBA (100/0 % (■), 75/25 % (■), 50/50 % (■), 25/75 % (■)) or with pluronic® F68 (□). For Caco-2/TC7 and HT-29/MTX cells only the concentrations 12.5 and 50 µg/mL were studied. Data are the mean of three determinations \pm sd. * significant versus control $p < 0.05$. Student test at 0.05 %. Low values of scale bars are included in the graphics can not be seen.

4.4. Evaluation of the toxicity of the formulations on vagina pig mucosa

The vagina is a fibromuscular tube with a wall consisting of three layers: the mucosa, the muscularis and the adventitia of the vagina. The mucosa is composed of a stratified squamous epithelium including a deep stratum basalis, an intermediate stratum spinosum, superficial layers of flat eosinophilic cells which contain keratin but which do not normally form a true horny layer. This stratified epithelium rests on a very cellular lamina propria including many leukocytes. Towards the muscularis some *vascular cavernous spaces* may be seen (typical erectile tissue).

The different formulations were deposited on the epithelium of pig vaginal mucosa and the architecture was observed after 24 h of contact to evaluate the toxicity. The results on Figure 10A showed that in the presence of SVF, the epithelium was regular with a totally preserved architecture. In contact with pluronic[®] F127 20 wt% the epithelium was regular and folded (Figure 10B). In the presence of NPs, the epithelium presented a regular architecture with a slight focal desquamation without stroma abnormalities (Figure 10C). In the presence of NPs jellified with pluronic[®] F127 hydrogel 20 wt% a regular and slightly basophilic epithelium with a preserved architecture was observed (Figure 10D).

On all specimens, true ulcerations were absent and the underlying stroma was devoid of inflammatory cells which confirmed the absence of significant toxicity.

The NPs Chito20/Chito20-TBA (75/25 %) jellified or not with pluronic[®] F127 did not appear to be toxic to healthy epithelium.

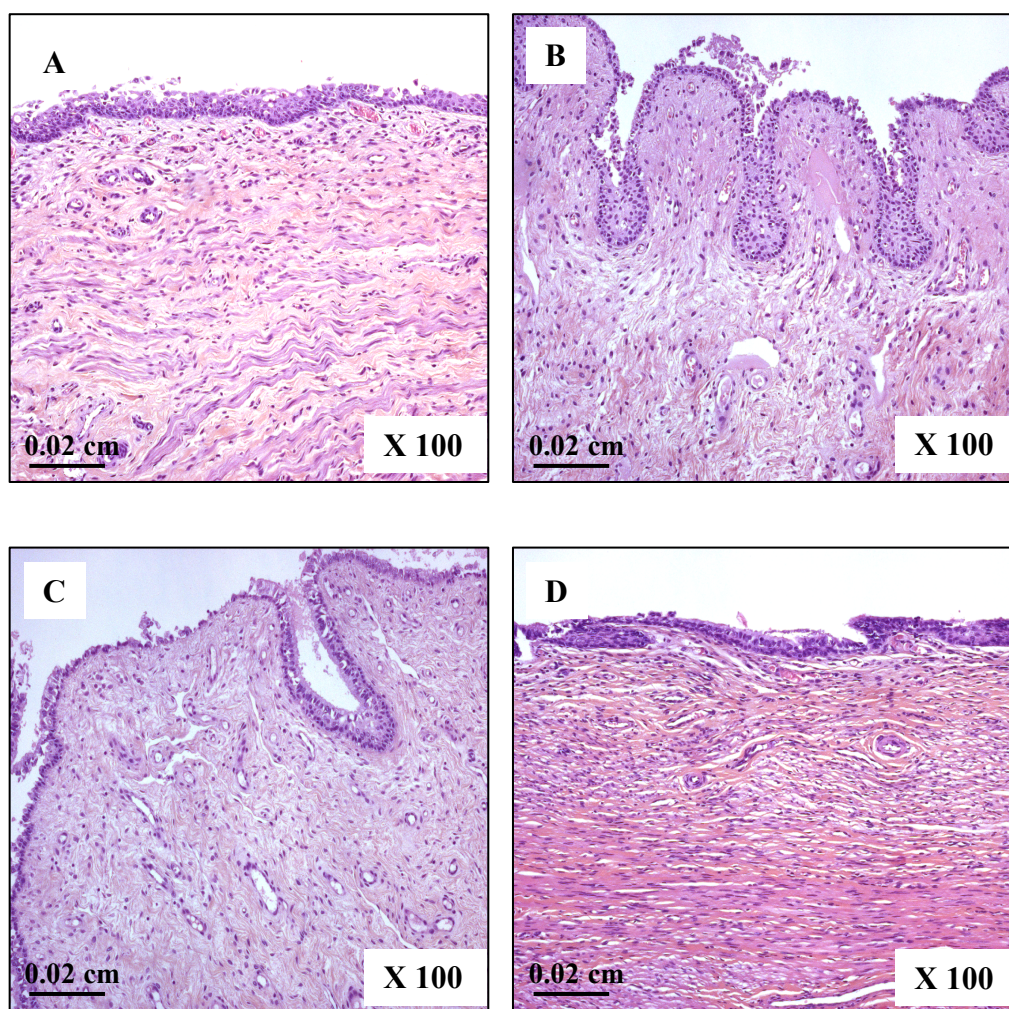
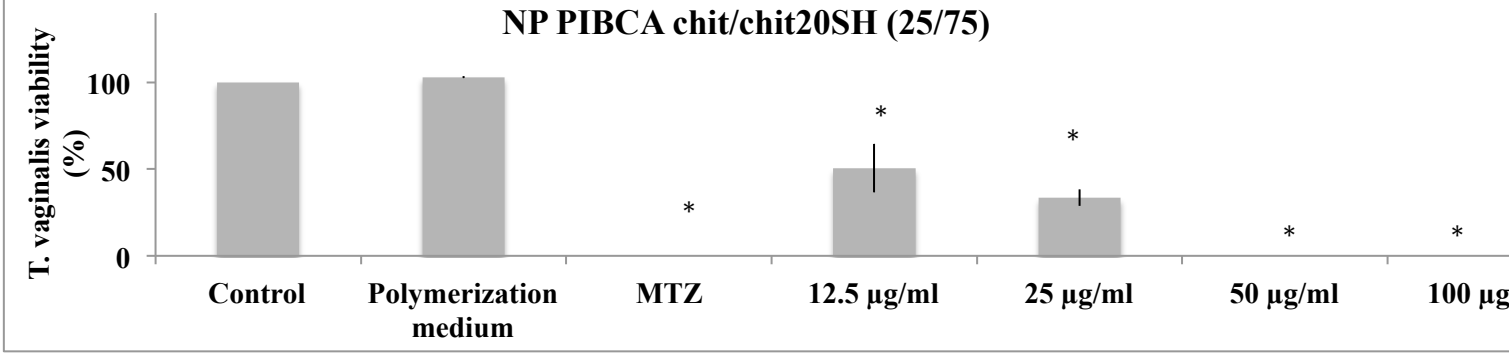
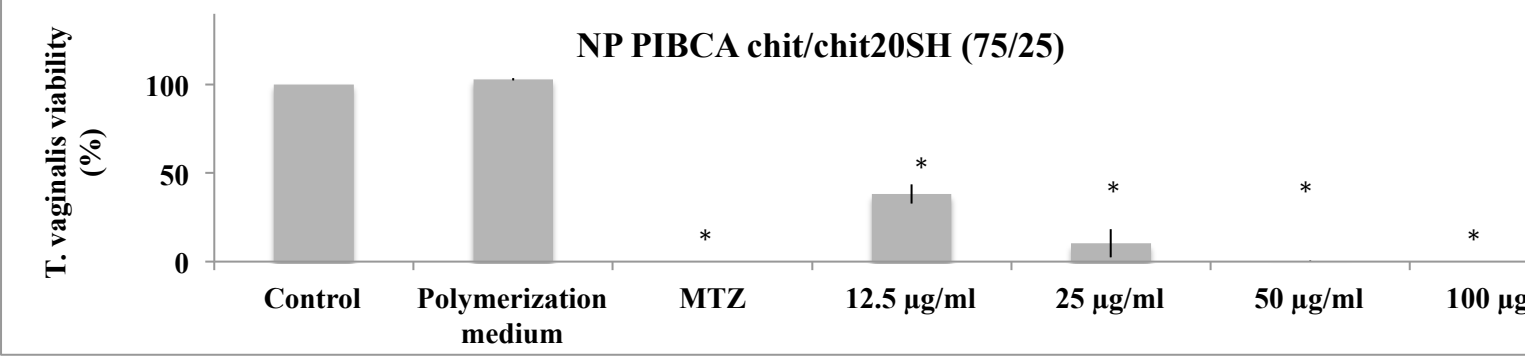
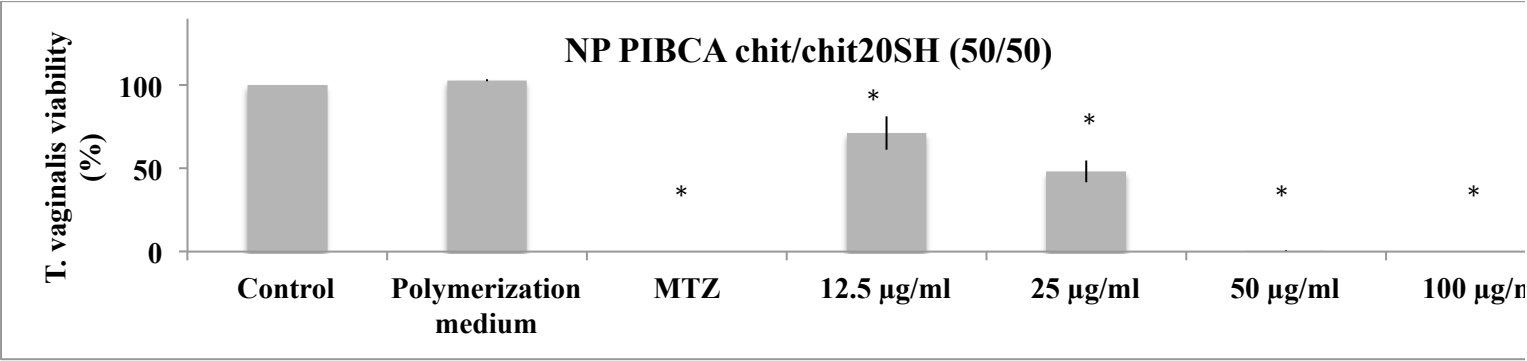
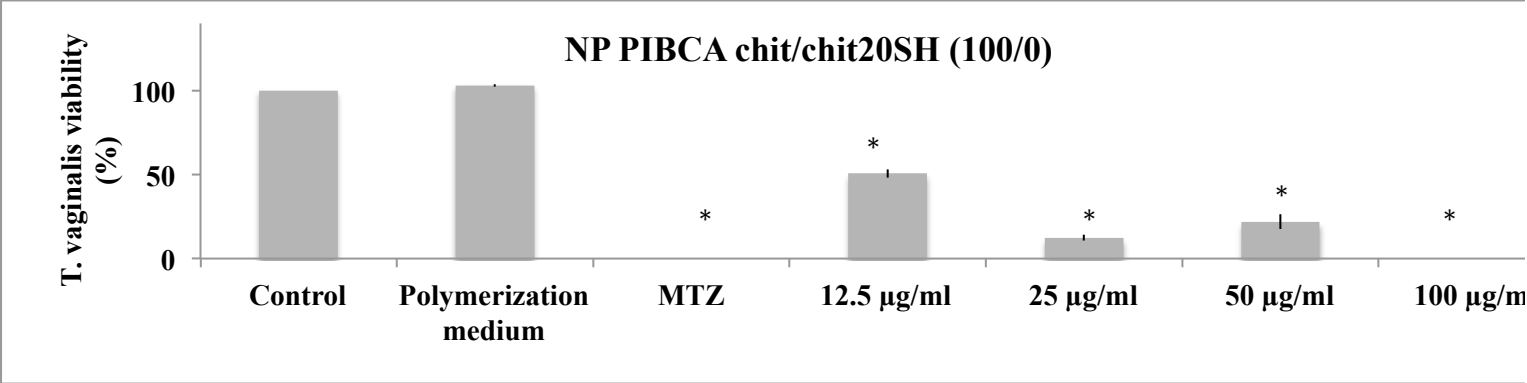


Figure 10. Example of histology images represented pig vaginal mucosa after 12 h in contact with SVF (A), F127 20 wt% (B), NPs Chito20/Chito20-TBA (75/25 %) (C) or PIBCA/(Chito20/Chito20-TBA) NPs (75/25 %) gelified with F127 20 wt% (D).

4.5. Evaluation of the anti-*T. vaginalis* activity of the formulations

The trichomonacidal activity of the NPs composed of PIBCA/(Chito20/Chito20-TBA) (75/25 %) was studied after 24 h of direct contact with *T. vaginalis*. The results were gathered on Figure 11.

After 24 h of incubation, even at low concentrations, the PIBCA/(Chito20/Chito20-TBA) NPs exhibited a trichomonacidal effect. However the rate of thiol has no effect on this activity. The PIBCA/(F68) showed no trichomonacidal effect.



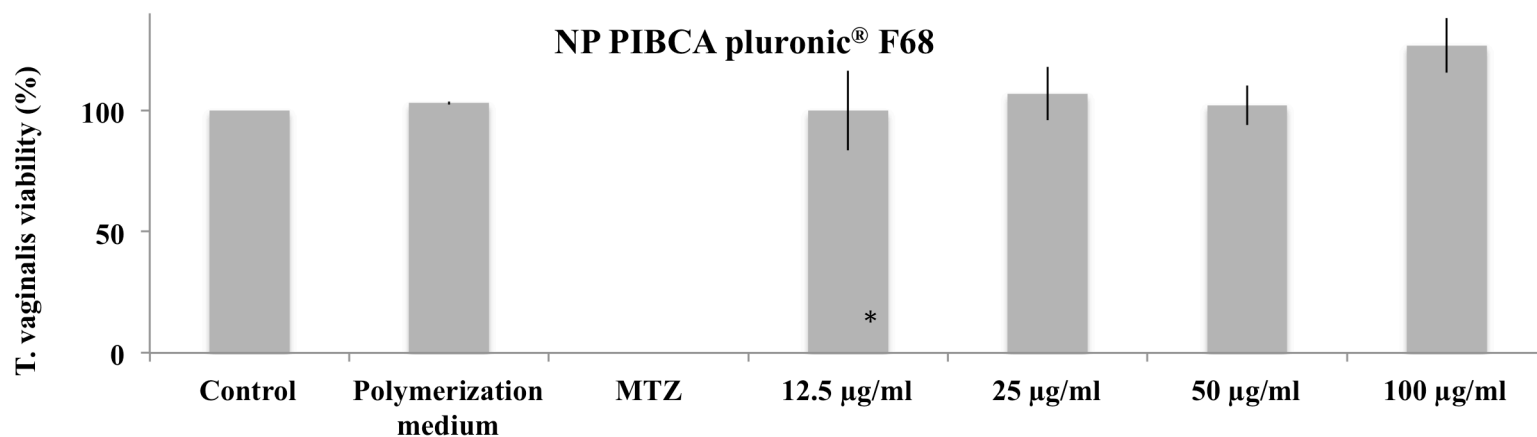


Figure 11. *Trichomonas vaginalis* (*Tv*) viability after a 24 h incubation period with different concentration of NPs PIBCA/(Chito20/Chito20-TBA) (100/0 %), (75/25%), (50/50 %) et (25/75%) and NPs PIBCA/pluronic® F68. *T. vaginalis* in culture medium without NPs was used as negative control. MTZ was at a concentration of 7 mg/ml. Data are the mean of three determinations \pm sd. * significant versus control $p < 0.05$.

5. Conclusions

In conclusion, PIBCA NPs dispersed into pluronic® F127 loose a part of their components which formed mixed micelles with pluronic® F127 monomer. This phenomenon prevents the thermoreversibility of the hydrogel and changes its physico-chemical properties. The polymerization duration is an important parameter and the NPs polymerized during 24 h seemed to be more stable than the NPs polymerized during only 1 h. The inclusion of NPs in pluronic® F127 20 wt% hydrogel will not be a problem for its use as well as the thermoreversibility was affected the gelling temperature remains below body temperature. However NPs were immediately degraded in the hydrogel and it posing a problem about the gradual release of active molecules that may encapsulate.

The NPs present a very important toxicity against HeLa cells but no toxicity was showed against Caco-2/TC7 and HT-29/MTX. Moreover, histological investigation did not show any NP toxicity against pig vaginal mucosa. Interestingly, PIBCA/(Chito20/Chito20-TBA)

(75/25 %) NPs show high trichomonacid activity. These results constitute a starting point for further biological evaluations of these formulations.

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Chapitre 2: The unexpected increase of clotrimazole
apparent solubility using randomly methylated β -
cyclodextrin

The unexpected increase of clotrimazole apparent solubility using randomly methylated β -cyclodextrin

Bénédicte PRADINES^{a,b}, Jean-François GALLARD^c, Bogdan I. IORGA^c, Claire GUEUTIN^a,
Gilles PONCHEL^a Philippe M. LOISEAU^b, Kawthar BOUCHEMAL^{a*}

a- Institut Galien Paris Sud, UMR CNRS 8612, Université Paris-Sud, Faculté de Pharmacie, 5, rue J-B. Clément, 92296 Châtenay-Malabry cedex, France.

b- Université Paris-Sud, UMR CNRS 8076 Laboratoire « Biomolécules : conception, isolement, synthèse » - « Chimiothérapie Antiparasitaire » Faculté de Pharmacie, 5, rue J.B. Clément, 92296 Châtenay-Malabry cedex, France.

c- Institut de Chimie des Substances Naturelles, CNRS UPR 2301, Centre de Recherche de Gif-sur-Yvette, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette, France

*Corresponding author: kawthar.bouchemal@u-psud.fr

France. Tel : +33 (0)1 46 83 55 81. Fax : +33 (0)1 46 61 93 34.

Abstract

Clotrimazole (CTZ) and cyclodextrin (CD) inclusion complexes having improved apparent water solubility were characterized from phase solubility diagrams. β -CD (1.5 % w/w) and hydroxypropyl- β -CD (HP- β -CD) (40 % w/w) offered poor CTZ solubility enhancements (12 and 384-time respectively). The apparent solubility of CTZ was 9980-time increased from $0.4 \mu\text{g.mL}^{-1}$ ($1.42 \mu\text{M}$) without CD to 4.89mg.mL^{-1} (14.9mM) using randomly-methylated β -CD (Me- β -CD) (40 % w/w). This was the highest apparent CTZ solubility improvement ever reported in the literature using conventional CDs. Quantitative nuclear magnetic resonance (^1H -NMR) coupled with two-dimensional nuclear overhauser effect (NOESY) experiments and molecular docking calculations showed that the highest interactions with Me- β -CD were reported for CTZ two phenyl groups. A lower interaction was reported for chlorophenyl, while imidazole had the weakest interaction with Me- β -CD. It is suggested that the simultaneous formation of these different complexes could explain the overall large apparent solubility increases of CTZ.

Keywords: Clotrimazole; cyclodextrins; solubilisation; bidimensionnal ^1H -RMN; molecular docking.

1. Introduction

Candida albicans is a commensal colonizer of mucous membranes that can become an opportunistic pathogen causing common mucosal infections as well as life threatening invasive infections in immunosuppressed patients (Odds et al., 1990). The infection caused by *Candida* species affects 70–75 % of women at least once during their lives and *Candida albicans* is responsible for 85-95 % of vaginal yeast infections (Sobel 2007). Patients with *Candida* vaginitis may not show any particular symptoms, but symptoms such as itching, burning, caseous discharge, painful sexual intercourse, and vulva edema and erythema were reported. Approximately 5-8 % of women of reproductive age with a primary episode of vulvovaginal candidiasis will develop recurrent disease (Hurley., 1981; Nyirjesy., 2001; Sobel., 2007). Moreover during pregnancy, asymptomatic candida colonisation of the vagina is common affecting 30–40 % of women. Neonates may acquire *Candida* species vertically through the vagina during labour (Filippidi et al., 2014). Oral chemotherapy is the major weapon available against *Candida albicans* vaginal infections; however, the success of this approach is undermined by widespread resistance to some of the most effective drugs ever developed. In addition, the use of the systemic administration of antifungal drugs such as fluconazole for prophylaxis or treatment of candidiasis infection is largely responsible for the rising problems of *Candida albicans* resistance.

So far, clotrimazole (CTZ) (Figure 1), has been used as an antimycotic drug by both local and systemic administration for the treatment of vaginal infections due to *Candida albicans*. The local administration of antimicrobial agents allows high concentrations to be reached close to the microbe. These concentrations are much higher than the ones reached by systemic administration (Buchholz et al., 1970). Furthermore, oral CTZ administration induces severe

hepatic toxicity (Miranda et al., 1998; Yong et al., 2007). However, the aqueous CTZ solubility is extremely low ($0.49 \mu\text{g.mL}^{-1}$ at 25°C) limiting thus the antifungal activity.

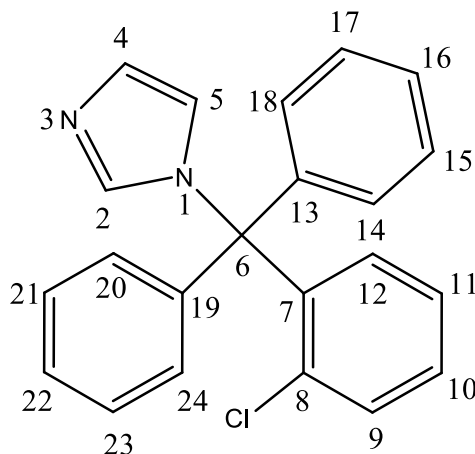


Figure 1: Chemical representation of CTZ.

In this context, one strategy to improve CTZ apparent solubility in pharmaceutical formulations is to use cyclodextrins (CDs). They are cyclic oligosaccharides that tend to form inclusion complexes. So far, complexation of poorly water-soluble drugs with CDs represents an interesting strategy for increasing their apparent water solubility (Duchêne et al., 1999) and offers further possibilities for their formulation (Loftsson and Duchêne 2007). CD/drug complexes can be much more water-soluble than the non-complexed drug. Only few research works reported the solubilisation of CTZ using CDs. α -cyclodextrin (Ahmed et al., 1998), β -cyclodextrin (β -CD) (Ahmed et al., 1998, Yong et al., 2007, Bilensoy et al., 2006), dimethyl- β -cyclodextrin (DiMe- β -CD) (Ahmed et al., 1998) or γ -cyclodextrin (Taneri et al., 2003) were used to improve CTZ apparent aqueous solubility.

In the work to be presented here, other types of CDs such as randomly methylated β -cyclodextrin (Me- β -CD) was used to increase the apparent water solubility of CTZ. This CD

has previously shown higher solubilisation behaviours in comparison with other types of CDs (Mazzaferro *et al.*, 2011). Up to now, little attention has been paid to the investigation of the interaction mechanism between CTZ with CDs. The chemical groups involved in this interaction are still unknown. In this context, an in depth physico-chemical methods including nuclear magnetic resonance (^1H -NMR) coupled with two-dimensional nuclear overhauser effect (NOESY) and molecular modeling were used to investigate the mechanism of the interaction between Me- β -CD and CTZ.

2. Materials and methods

2.1. Reagents

Clotrimazole (CTZ) ($M_w=344.37 \text{ g.mol}^{-1}$) was from Inresa Pharma (Bartenheim, France). β -CD ($M_w=1134.99 \text{ g.mol}^{-1}$) was from Wacker (München, Germany). Me- β -CD Rameb[®] ($M_w=1331.36 \text{ g.mol}^{-1}$) was from Cyclolab (Budapest, Hungary). Hydroxypropyl- β -cyclodextrin (HP- β -CD, $M_w=1375.36 \text{ g.mol}^{-1}$) was from Acros Organics (Geel, Belgium). All chemicals were of analytical or reagent grade and were used without further purification. Solutions were prepared by weight using MilliQ[®] water (Millipore, Molsheim, France). Analytical grade methanol (MeOH) was from Carlo Erba (Val de Reuil, France). Deuterium oxide (D_2O) (99.96 % D), methanol deuterium (MeOHD_4) were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

2.2. Phase solubility diagrams and apparent solubility determinations

Phase-solubility studies were carried out according to the method described by Higuchi and Connors (1965). An excess of commercial CTZ was placed in a glass vial (20 mL) with 2 mL of solubilizing agent which was an aqueous solution of CDs at increasing

concentrations ranging from 5 % to 40 % w/w for Me- β - or for HP- β -CD and from 0.5 to 1.5 % w/w for β -CD due to its own water solubility limitations at 25 °C.

Vials containing an excess of drug powder dispersed in a solution of CD were kept in a shaker during 5 days at 25 °C. This duration was estimated to be long enough for reaching a complexation equilibrium. Then, the samples were filtered through a 0.22 μ m membrane filter (Millex, SLAP 0225, Millipore, France).

The solubility of CTZ in water was determined according to the same protocol without the presence of CDs.

2.3. HPLC determination of CTZ concentrations

The samples containing CTZ were analyzed by reversed phase high performance liquid chromatography (HPLC) using a symmetry C₁₈ column (5 μ m, 250 x 4 mm, Waters). Chromatography was performed with a Waters 515 pump and a Waters 717Plus autosampler. UV absorbance at 230 nm was monitored with a Waters 486 absorbance detector. Data were recorded and processed on Azur Software (Datalys). The mobile phase was an isocratic mixture of methanol/water (75:25 % v/v). HPLC analyses were performed at a flow rate of 0.5 mL.min⁻¹.

The calibration curve was obtained from a stock solution of Me- β -CD/CTZ complex (250 μ g.mL⁻¹) prepared according to the method described in the previous section. The concentration of CTZ should be below the solubility of the inclusion complex. The stock solution was diluted with the Me- β -CD solution. Valid concentrations for quantification were in the range 0.25-250 μ g.mL⁻¹. The equation of the calibration curve was $y = 92.009 x + 252.960$ ($R^2 = 0.9997$).

The calibration curve was compared to the one obtained from a stock solution of CTZ dissolved in methanol at a concentration of $500 \mu\text{g.mL}^{-1}$. This calibration curve was obtained by diluting the stock solution with the mobile phase. Valid concentrations for quantification were in the range $0.25\text{-}500 \mu\text{g.mL}^{-1}$. The equation of the calibration curve was $y = 83.372 x + 590.64$ ($R^2 = 0.9998$) for CTZ in methanol.

A Student's t distribution test was carried out to compare the slope and the intercept of the two previous equations. The p-values were higher than 5 %, supporting that the linear least square lines could be considered statistically equal.

However, it was not possible to prepare sufficiently concentrated stock solutions of CTZ complexes prepared with HP- β -CD and β -CD due to the poor CTZ solubilisation effect obtained using these CDs.

According to the data, there was no influence of the presence of Me- β -CD on the valid calibration curves. Consequently, the concentrations of CTZ for β -CD and HP- β -CD were estimated from the calibration curve obtained with methanol.

2.4. Nuclear Magnetic Resonance (^1H -NMR) experiments

^1H -NMR spectra were obtained by operating at 600 MHz (Bruker Avance, TXI SB 5 mm) for the free CTZ, the inclusion complex Me- β -CD/CTZ and the Me- β -CD alone. The two-dimensional nuclear Overhauser effect (NOESY) spectra of the complex were performed with a Bruker Avance, TXI Cryoprobe at 600 MHz.

The inclusion complex of CTZ and Me- β -CD (10 % w/w) for NMR analyses was prepared as described in section 2.2 by replacing water by D_2O . Free CTZ solutions were prepared by placing an excess of commercial CTZ powder in a glass vial (20 mL) with either 2 mL of D_2O or by dissolving CTZ in MeOH-D_4 at a concentration of 1 mg.mL^{-1} . Then, the

samples were filtered through a 0.22 μm membrane filter (Millex, SLAP 0225, Millipore, France). Chemical shifts were reported in ppm (δ) downfield from tetramethylsilane (TMS) internal reference.

2.5. Molecular modeling

To gain insight into the interaction between CTZ and Me- β -CD, the molecular docking technique was used. The Me- β -CD structure used in this work was generated from the curated coordinates of ligand QKH (structure 2QKH, Parthier et al., 2007), downloaded from HIC-Up database (Kleywegt, 2007), by manually adding methyl groups on all free hydroxyl groups in order to obtain a fully permethylated structure (GaussView 5, Semichem Inc). All dihedral angles of the methoxy groups were then homogenized, the resulting conformations being compatible with an unhindered CD cavity. Three-dimensional coordinates of CTZ were generated using Corina version 3.44 (<http://www.molecular-networks.com/>). CD dimers were generated from two identical monomers aligned on the cavities' axes, with the outer sides pointing one to another. As the optimal distance between CD monomers is not known, eleven dimers were generated, with distances between cavities' centers ranging from 10 Å to 20 Å, which were then used as receptors in the docking procedure.

Molecular docking was carried out using Gold (Verdonk et al., 2003) and the GoldScore scoring function. The binding site was defined as a sphere with a radius of 20 Å centered on the cavity center for the 1:1 complex and on the half-distance between cavities centers for the 2:1 complexes. All other parameters were used with the default values.

Surface calculations were carried out using the *g_sas* module as implemented in the Gromacs 4.6.1 software package (Pronk et al., 2013), using 0.1 nm as radius for the solvent probe and a number of 1 000 dots per sphere.

Molecular modeling figures were generated using PyMol (<http://www.pymol.org/>).

3. Results and discussion

Despite the fact that CTZ has proven to be efficient against candidiasis, its extremely low aqueous solubility represents an obstacle from a formulation point of view. The use of CDs represents an interesting strategy for the improvement of CTZ apparent aqueous solubility in physiological fluids. The influence of the CD substituents of β -CD (β -, HP- β - and Me- β -CD) on the apparent solubility of CTZ in water was investigated by phase solubility diagrams. The examination of the obtained diagrams on Figure 2 gave an overview of the process of complexation between ABZ and the CDs based on Higuchi and Connors' classification (Higuchi and Connors, 1965). "A" type curves were obtained whatever the CD used indicating the formation of water soluble complexes on the whole range of CD concentrations investigated. Type "A" diagrams are represented by a linear increase in solubility " A_L ", or by a linear increase with a positive or negative deviation from linearity " A_p " and " A_N " respectively (Figure 2.B).

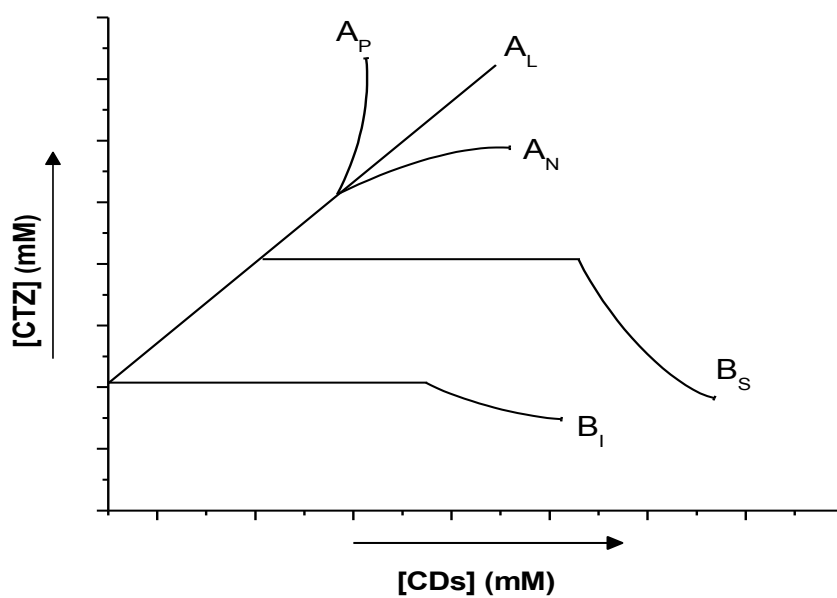
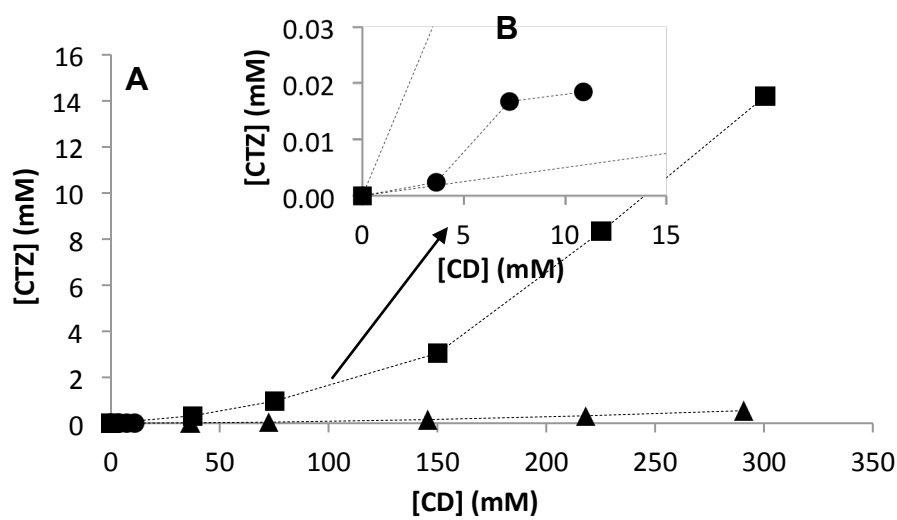


Figure 2. (A) Experimental phase solubility diagram of CTZ for different CDs ($n = 3$). (●) β -CD, (▲) HP- β -CD and (■) Me- β -CD. (B) Typology of phase solubility diagrams according to (Higuchi and Connors 1965), representing: “A”: soluble inclusion complex. “B”: formation of insoluble inclusion complex. “ A_L ”: linear increase of drug solubility as a function of CD concentration. “ A_P ”: positively deviated curve. “ A_N ”: negatively deviated curve. “ B_S ”: complex with limited solubility. “ B_I ”: insoluble complex.

Table 1. Highest observed apparent solubilities of CTZ in different CD solutions in water. The apparent solubility enhancement factors (F) were obtained by dividing the solubility of CTZ in presence of CDs by the solubility of CTZ in water without CD*. $K_{1:1}$ is the apparent stability constant calculated from the slope of the phase solubility diagrams according to equation 1. $T^\circ = 298\text{ K}$ ($25\text{ }^\circ\text{C}$), $n = 3$.

CD	CD		Maximal apparent Solubility of CTZ		F	$K_{1:1}$ (M^{-1})
	% (w/w)	(mM)	(mg.mL^{-1})	(mM)		
None*	0	0	0.00049	0.00142	1	-
β -CD	1.5	10.90	0.01 ± 0.07	0.01 ± 0.06	12	-
HP- β -CD	40	290.83	0.18 ± 0.01	0.54 ± 0.01	384	875
Me- β -CD	40	300.44	4.89 ± 0.02	14.18 ± 0.01	9982	-

A linear increase in CTZ solubility as a function of CD concentration was obtained with HP- β -CD (type “ A_L ”). The A_L diagram is observed if all inclusion complexes are of the first order in CD. The A_L type diagram does not mean necessarily that only 1:1 complex is formed. This diagram means that only one HP- β -CD is involved in the interaction with one or more molecule of CTZ. They may be written HP- β -CD/CTZ₁, HP- β -CD/CTZ₂, HP- β -CD/CTZ₃.... In the absence of additional information on the inclusion of CTZ with HP- β -CD, the assumption of a 1:1 complex is usually made. In this case, the stability constant (also called complex formation constant or complex equilibrium constant) of CTZ with HP- β -CD was calculated according to the equation 1 (Grant and Higuchi., 1990):

$$K_{1:1} = \frac{\text{Slope}}{S_0(1 - \text{Slope})} \quad \text{Equation 1}$$

Where S_0 is the equilibrium solubility of CTZ in the absence of CD.

Assuming a 1:1 stoichiometry the calculated stability constant for HP- β -CD was 875 M^{-1} .

The apparent solubility enhancement of CTZ was low using β -CD (up to 0.018 mg.mL^{-1}), while HP- β -CD 40 % w/w allowed to reach a CTZ concentration of 0.546 mg.mL^{-1} . It is worth to note that these apparent solubility enhancements are different from the ones reported in the literature for CTZ. Enhancements factors of 3.8 and 5.9 were obtained for β -CD and HP- β -CD respectively using another solubilisation technique (Taneri et al., 2003). In this method, a mixture of 0.10 g CTZ and 0.90 g of different CDs was diluted to 25.0 g with distilled water and then stirred for only 2 h with a magnetic stirrer. Resulting suspensions were filtered through filter papers and the concentration of CTZ was evaluated (Taneri et al., 2003). However, while the initial interaction between a molecule and a CD to form the complex is very rapid (often within minutes), the final equilibrium can take much longer to reach (generally, about few days) (Loftsson et al., 2002).

The highest increase of apparent CTZ solubility was obtained using Me- β -CD 40 % w/w (4.89 mg.mL^{-1}). This was the highest increase of CTZ apparent solubility ever reported in the literature using conventional CDs. The phase-solubility curve of CTZ when Me- β -CD was used allowed to A_P type curve. In this light, the A_P diagram suggests that the inclusion complexes were present to a higher order than one CTZ per Me- β -CD. These complexes may be written Me- β -CD₁/CTZ, Me- β -CD₂/CTZ, Me- β -CD₃/CTZ...More precisely, the phase-solubility diagram of Me- β -CD was divided in two parts: the first one ranged from 0 to 150 mM and the second one from 150 mM to 300 mM.

One hypothesis is that one molecule of CTZ interacted with one molecule of Me- β -CD for the first part of the curve, while one molecule of CTZ interacted with two molecules of Me- β -CD for the second part of the curve. In this latter part of the curve, it was thus possible to postulate that two Me- β -CDs molecules interacted with one CTZ molecule. To gain insights on the complexation mechanism between Me- β -CD and CTZ, $^1\text{H-NMR}$ (NOESY) and

molecular modelling were used. A first set of experiments was carried out by ^1H -NMR in order to compare the spectrum of the Me- β -CD/CTZ complex with the one of the free drug and Me- β -CD. Low concentration of the Me- β -CD (10 % w/w instead of 40 % w/w) was preferred for the NMR characterization.

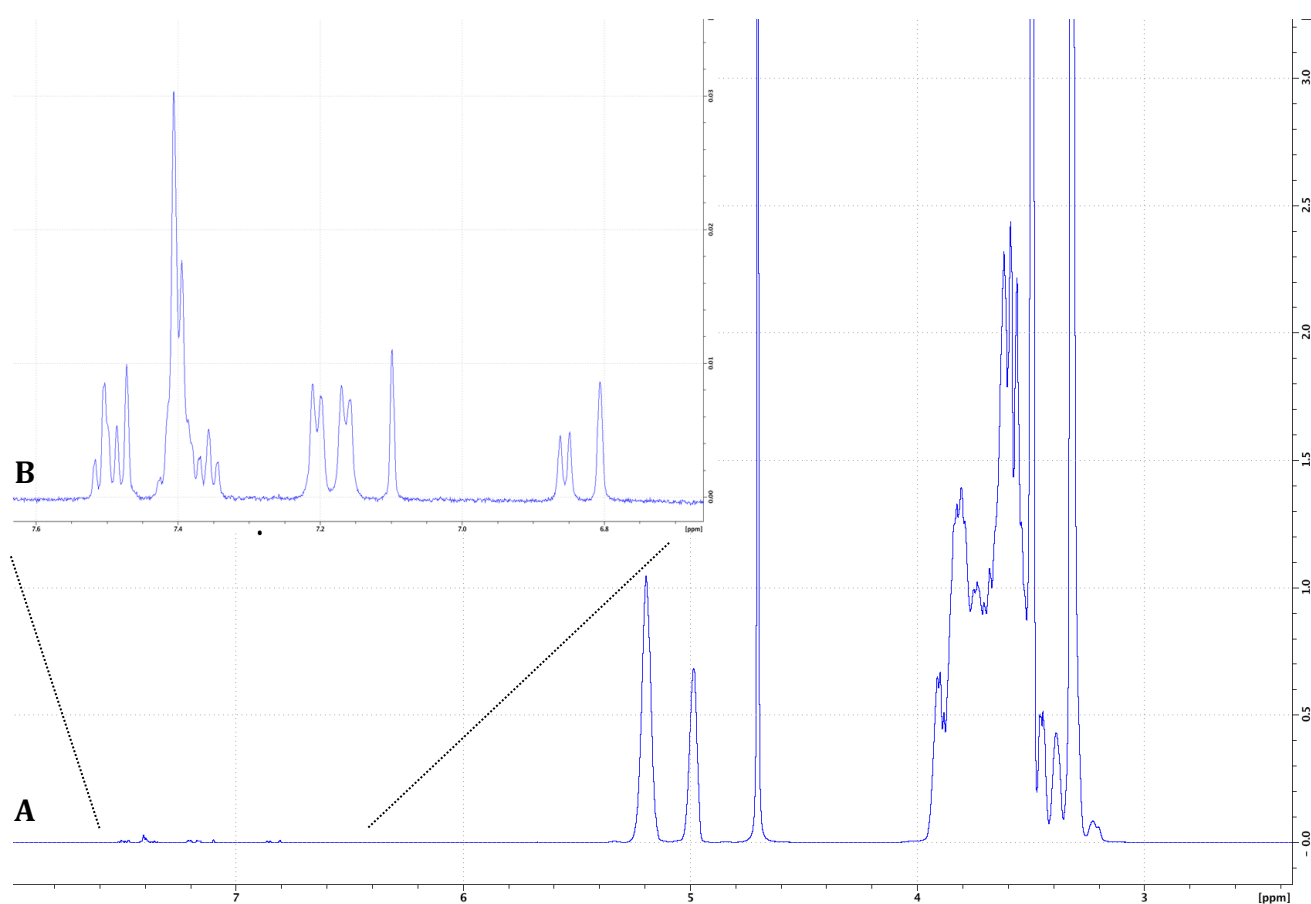


Figure 3: (A) ^1H NMR spectrum of Me- β -CD/CTZ inclusion complex in D_2O (600 MHz, NS=64, $[\text{CTZ}]=0.25 \text{ mg.mL}^{-1}$). (B) Represents a zoom of the region corresponding to CTZ displacements. The concentration of Me- β -CD was 10 % w/w.

The NMR spectrum of the inclusion complex of Me- β -CD with CTZ presented on Figure 3 showed the presence of both molecules. Obviously, the peak intensity was too much higher for Me- β -CD (Figure 3.A) in comparison with CTZ (Figure 3.B) because the concentration of the CD was higher than the one of CTZ.

On the other hand, the chemical shifts of the peaks obtained in this region for non-complexed CTZ (Figure 4.A) were then compared to the ones for CTZ complexed with Me- β -CD (Figure 4.B). Noteworthy, ^1H -NMR spectra of non-complexed CTZ were first investigated in D_2O . However, difficulties were found to obtain a spectrum using D_2O due to the limited solubility of CTZ in water (data not shown). Deuterated methanol was thus used.

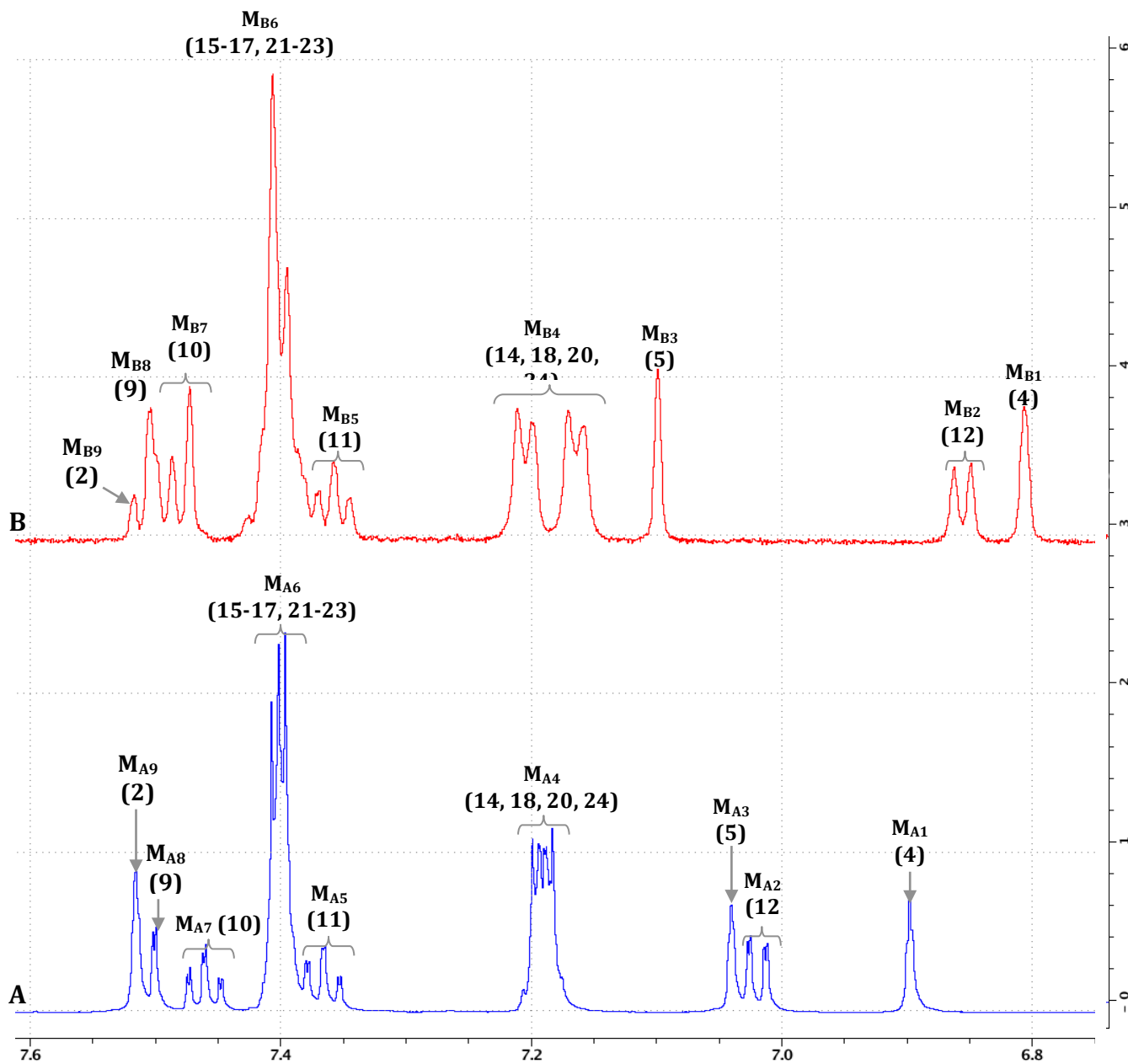


Figure 4: ^1H NMR spectra of (A) non-complexed CTZ in MeOD (600 MHz, NS=64, [CTZ]=1 mg.mL $^{-1}$) and (B) Me- β -CD/CTZ inclusion complex in D₂O (600 MHz, NS=64, [CTZ]=0.25 mg.mL $^{-1}$).

Table 2: ^1H NMR band assignments of (A) non-complexed CTZ and (B) inclusion complex of Me- β -CD/CTZ.

Atom	(A) Non-complexed CTZ		(B) Inclusion complex Me- β -CD/CTZ	
	Multiplet	Shift (ppm)	Multiplet	Shift (ppm)
4	M _{A1}	6.89	M _{B1}	6.79 – 6.82
12	M _{A2}	7.01 - 7.03	M _{B2}	6.84 – 6.88
5	M _{A3}	7.04	M _{B3}	7.09 – 7.11
14, 18, 20, 24	M _{A4}	7.16 - 7.21	M _{B4}	7.14 – 7.23
11	M _{A5}	7.34 - 7.38	M _{B5}	7.33 – 7.38
15, 16, 17, 21, 22 and 23	M _{A6}	7.38 - 7.42	M _{B6}	7.38 – 7.44
10	M _{A7}	7.44 - 7.48	M _{B7}	7.46 – 7.49
9	M _{A8}	7.49 - 7.50	M _{B8}	7.49 – 7.51
2	M _{A9}	7.51 - 7.52	M _{B9}	7.51 – 7.52

The region from 6.8 to 7.6 ppm corresponding to non-complexed CTZ and its inclusion complex with Me- β -CD are presented on Figures 4.A and 4.B. Details on the peak multiplets and the corresponding chemical shifts are shown on Table 2. The chemical groups of CTZ involved in the interaction with Me- β -CD cavity were then studied by two dimensional Nuclear Overhaused Effect measurements (^1H -NMR 2D NOESY). Knowing that all the chemical shifts of Me- β -CD were localized in the region 3.2 - 4.15 ppm (Figure 3.A) and the ones corresponding to CTZ ranged from 6.8 to 7.6 ppm (Figure 3.B), the analysis of the spectra was limited to these two regions.

Figure 5 showed the cross peak NOESY bands that coincided with the intermolecular interactions between the CTZ protons and the internal protons of the Me- β -CD cavity. The intensity of the peaks was high for all the phenyl protons on the carbons C₁₄-C₁₈, C₂₀-C₂₄ (supporting information). An interaction was also reported for chlorophenyl protons on the carbons C₉ and C₁₀ but the intensity was lower than the one reported with phenyl groups (see supplementary data). Finally, the lowest peak intensity corresponded to the interaction with the protons of imidazole group on the carbons C₂, C₄ and C₅.

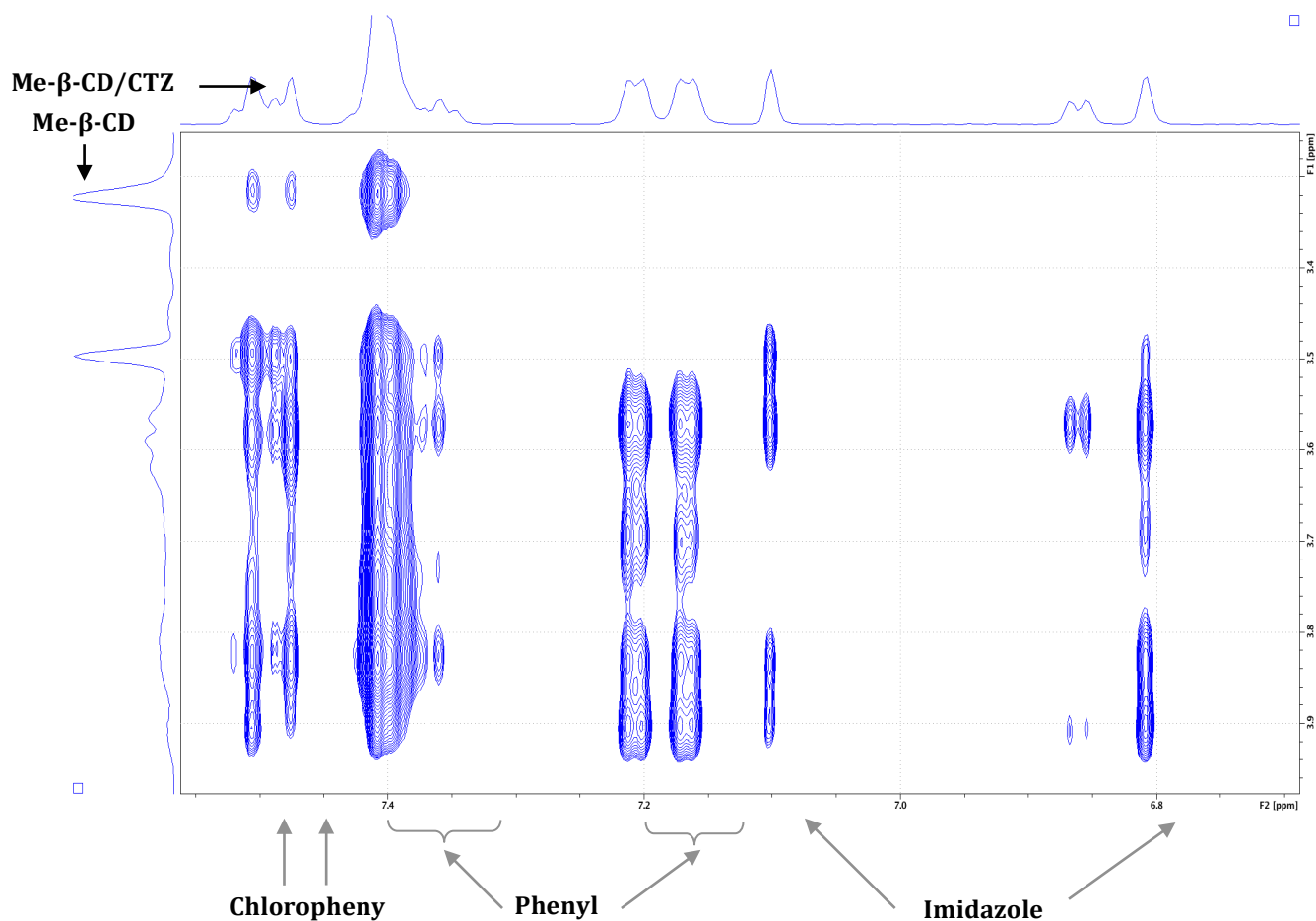


Figure 5: Two-dimensional spectrum (^1H NMR 2D NOESY) of Me- β -CD/CTZ inclusion complex (600 MHz, D_2O). The largest cross peak coincided with the intermolecular interactions between the hydrogen of CTZ and the internal hydrogens of the Me- β -CD cavity.

Molecular modelling was then used for an in-depth characterization of the interaction between CTZ and Me- β -CD. Molecular docking calculations showed that only one aromatic ring of CTZ was able to enter inside the Me- β -CD cavity (in 1:1 stoichiometry), while the second phenyl, the chlorophenyl and the imidazole groups were found to interact with the outer side of Me- β -CD molecule (Figures 6.A and 6.B). However, although this complex “hidden” about 59 % of the CTZ surface (233 out of 396 \AA^2), a significant amount of this surface is still solvent accessible (Figure 6.B). In these conditions, a Me- β -CD/CTZ interaction of type 2:1 cannot be excluded. To test this hypothesis, and as the optimal distance between two Me- β -CD molecules is not known, cavities of different sizes were generated

from two identical Me- β -CD molecules with the outer sides pointing one to another, aligned on the cavities axes, with distances between cavities centers ranging from 10 Å to 20 Å. This orientation is supposed to provide maximum host-guest contact surface and minimum steric hindrance between the Me- β -CD monomers. CTZ was docked in these cavities and in this case the optimum distance between the centers of monomers was found to be 10 Å. This Me- β -CD/CTZ 2:1 complex, represented in Figure 6.C and 6.D, shows the same positioning of CTZ in the cavity as in the 1:1 complex, as well as an additional hydrogen bond between the imidazole ring and an oxygen atom from the outer cavity of the second Me- β -CD monomer. This 2:1 complex “hidden” about 72 % of the CTZ surface (286 out of 396 Å²) and therefore is considered to be more stable. However, an equilibrium between the 2:1 and 1:1 forms is also possible, the interaction between Me- β -CD and CTZ being by nature a dynamic process.

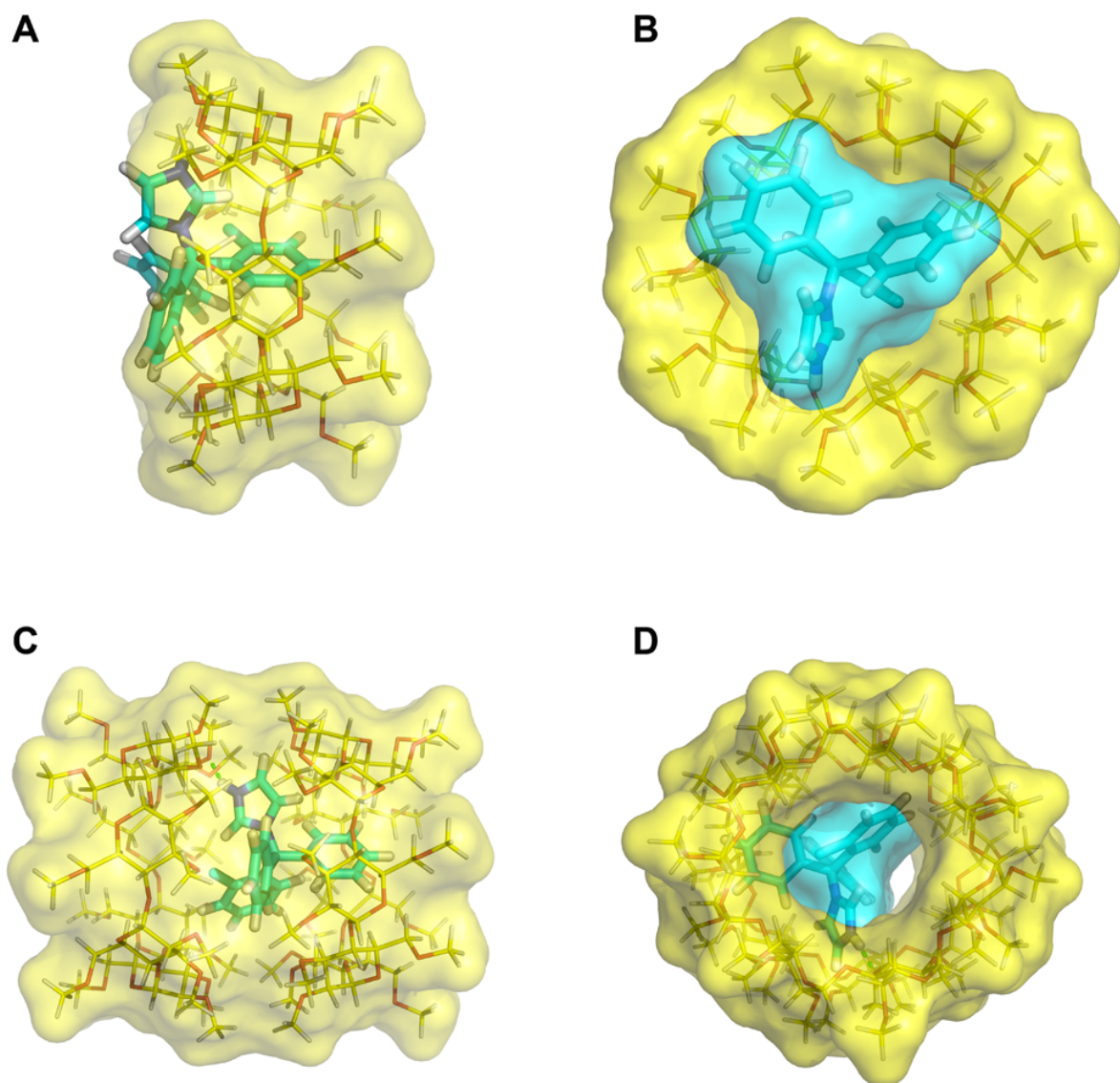


Figure 6: Representation of the complex of Me-β-CD (yellow) with CTZ (cyan) obtained by molecular docking with 1:1 (A, B) and 2:1 (C, D) stoichiometry. Lateral view of the monomer (A) and of the dimer (C), from the outer side of the monomer (B) and from one of the narrow sides of the dimer (D).

Consequently, a sequential two-step binding mechanism was found to be more suitable for describing the interaction of CTZ with Me-β-CD. This model is adapted to describe interactions for systems presenting non-identical sites. The two sets of site model assumes that the two interactions occur simultaneously and independently one from the other. In the sequential binding model, the first Me-β-CD₁ molecule which binds to CTZ always binds to

site 1, then the second Me- β -CD₂ molecule which binds to CTZ always binds to sites 2. Thus, CTZ exhibited four interacting groups, from which two distinct binding groups (one phenyl and one among the remaining phenyl, chlorophenyl or imidazole groups could sequentially bind to two Me- β -CD molecules. These series of complex events was likely to explain the observed dilatant A_p profiles in Higuchi diagram.

5. Conclusion

Among the different CDs used to improve the apparent aqueous solubility of CTZ, Me- β -CD allowed to reach the highest solubility enhancement ever reported in the literature for CTZ by using conventional CDs. The combination of solubility experiments to ¹H-NMR-NOESY and molecular docking calculations allowed to identify a bivalent sequential binding mechanism. A first molecule of Me- β -CD interacted with phenyl groups (C₁₄-C₁₈ and C₂₀-C₂₄), then, a second Me- β -CD molecule interacted with both chlorophenyl (C₉ and C₁₀) and imidazole (C₂, C₄ and C₅) groups of CTZ. This type of binding has never been reported before in the literature for CTZ and CDs. The results reported in this work constitute useful information for both fundamental and applied pharmacy.

6. Acknowledgments

This work was supported by grants from Région Ile de France.

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Supplementary data

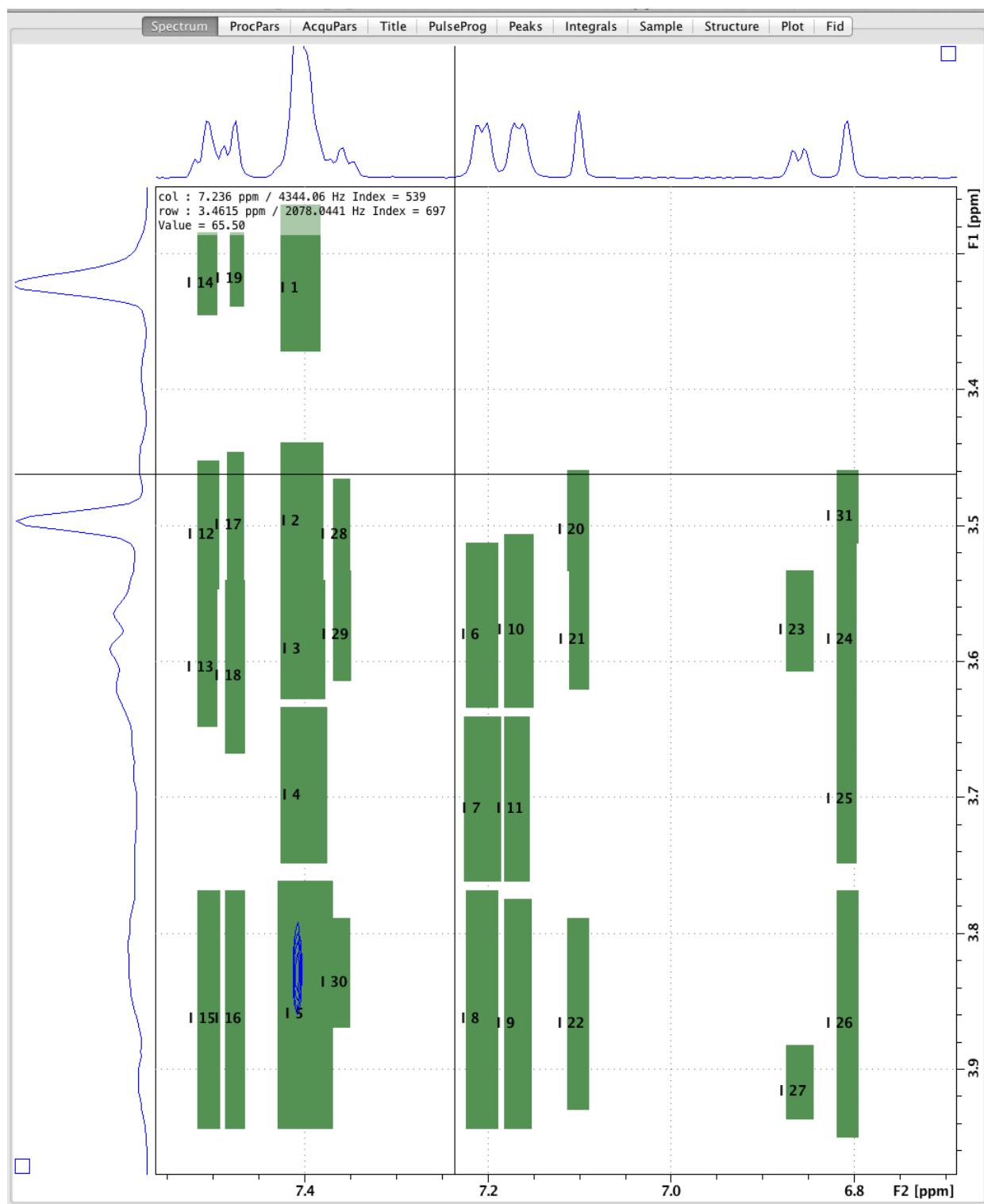


Figure 1: Two-dimensional spectrum (¹H-NMR 2D NOESY) of Me-β-CD/CTZ inclusion complex (600 MHz, D₂O). The largest cross peak coincided with the intermolecular interactions between the hydrogen of CTZ and the internal hydrogens of the Me-β-CD cavity.

Table 1: Integrals of the two-dimensional spectrum (^1H -NMR 2D NOESY) of Me- β -CD/CTZ inclusion complex (600 MHz, D_2O).

Integral number	Integral intensity (absorbance)	Integral relative intensity	δ_x (ppm)	δ_y (ppm)
1	536240	0.1754	7.4042	3.3172
2	693630	0.2269	7.4025	3.4886
3	949990	0.3107	7.4016	3.5827
4	1427300	0.4669	7.4016	3.6903
5	3057100	1.000	7.3991	3.8516
6	477850	0.1563	7.2067	3.5727
7	336650	0.1101	7.2059	3.7004
8	586350	0.1918	7.2067	3.8550
9	614470	0.2010	7.1681	3.8583
10	459910	0.1504	7.1672	3.5693
11	272420	0.0891	7.1681	3.7004
12	248760	0.0814	7.5050	3.4987
13	183480	0.0600	7.5058	3.5962
14	80640	0.0264	7.5058	3.3139
15	367460	0.1202	7.5042	3.8550
16	274900	0.0899	7.4765	3.8550
17	132270	0.0433	7.4756	3.4920
18	282660	0.0925	7.4756	3.6029
19	53666	0.0176	7.4747	3.3105
20	93042	0.0304	7.1017	3.4954
21	140260	0.0459	7.1008	3.5760
22	148920	0.0487	7.1017	3.8583
23	157620	0.0516	6.8605	3.5693
24	220340	0.0721	6.8084	3.5760
25	124030	0.0406	6.8084	3.6937
26	370350	0.1211	6.8084	3.8583
27	76583	0.0251	6.8597	3.9088
28	78741	0.0258	7.3605	3.4987
29	114620	0.0375	7.3588	3.5727
30	96589	0.0316	7.3596	3.8281
31	47508	0.0155	6.8084	3.4853

Chapitre 3: Investigation of the complexation of
albendazole with cyclodextrins for the design of new
antiparasitic formulations

Investigation of the complexation of albendazole with cyclodextrins for the design of new antiparasitic formulations

Bénédicte Pradines^{a,b}, , Jean-François Gallard^c, Bogdan I. Iorga^c, Claire Gueutin^a, Philippe M. Loiseau^b, Gilles Ponchel^a, Kawthar Bouchemal^{a*}

a- Institut Galien Paris Sud, UMR CNRS 8612, Université Paris-Sud, Faculté de Pharmacie, 5, rue J-B. Clément, 92296 Châtenay-Malabry cedex, France.

b- Université Paris-Sud, UMR CNRS 8076 Laboratoire « Biomolécules : conception, isolement, synthèse » - « Chimiothérapie Antiparasitaire » Faculté de Pharmacie, 5, rue J.B. Clément, 92296 Châtenay-Malabry cedex, France.

c- Institut de Chimie des Substances Naturelles, CNRS UPR 2301, Centre de Recherche de Gif-sur-Yvette, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette, France

Abstract

Albendazole (ABZ) exhibits a potent antiparasitic activity against a broad spectrum of parasites. Unfortunately, the very low water solubility of ABZ ($0.2 \mu\text{g.mL}^{-1}$ ($0.7 \mu\text{M}$)) impairs considerably its formulation. Phase solubility diagrams showed that α -cyclodextrin (10 % w/w), HP- β -cyclodextrin (40 % w/w) and SBE- β -cyclodextrin (40 % w/w) allowed an increase of apparent solubility with enhancement factors of 570, 3970 and 5880 respectively. The apparent aqueous solubility of ABZ was markedly increased from $0.2 \mu\text{g.mL}^{-1}$ ($0.7 \mu\text{M}$) without cyclodextrins to 1.52 mg.mL^{-1} (5.69 mM) with Me- β -cyclodextrin (40 % w/w). This corresponds to an apparent solubility enhancement factor of 7600 which is the maximal enhancement factor of ABZ apparent aqueous solubility ever reported in the literature using cyclodextrins. The complexation mechanism between ABZ and cyclodextrins has been investigated using phase solubility diagrams, nuclear magnetic resonance (^1H NMR) coupled with two-dimensional nuclear overhauser effect (NOESY) experiments and molecular docking calculations. The results showed that the central bicyclic fragment from ABZ interacts with Me- β -cyclodextrin according to 1:1 stoichiometry.

Keywords: Albendazole; cyclodextrins; solubilisation studies; bidimensionnal ^1H -RMN; molecular docking.

1. Introduction

Albendazole (ABZ), (methyl[5-(propylthio)-1H-benzimidazol-2-yl]carbamate (Fig.1), belongs to the group of benzimidazole derivatives. This molecule has a broad spectrum of activity against human and animal helminthic parasites such as nematodes, and metacestodes. It was approved for human use since 1982. ABZ showed antiprotozoal activity against *Trichomonas vaginalis* (Upcroft and Upcroft., 2001); *in vitro*, the IC₅₀ of ABZ against *T. vaginalis* was estimated in a range of 3-4 μM (Navarrete-Vazquez *et al.*, 2006). ABZ was also used to boost the trichomonacidal activity of metronidazole (Goodhew and Scor., 2013). However, like other benzimidazole carbamates, ABZ belongs to biopharmaceutical classification system type II (BCS class II) with low aqueous solubility ($0.2 \mu\text{g.mL}^{-1}$ at 25°C) and high permeability through the biological membranes.

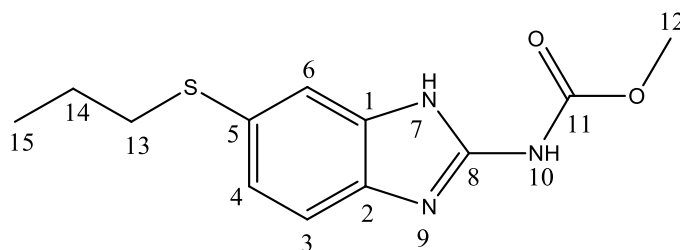


Figure 1. Chemical structure of ABZ.

Low water solubility of drugs results very generally in low solubility in biological fluids and is likely to considerably impair either attainable local drug concentrations when local delivery is foreseen or pharmacokinetic profiles after systemic delivery. Different approaches have been previously investigated to solve the aqueous solubility limitation of ABZ, including the use of surfactants such as polysorbates (Torrado *et al.*, 1996), bile salts (Estal *et al.*, 1993) or co-solvent like molecules (diethylene glycol monoethyl ether: Transcutol[®] (Torrado *et al.*, 1997). High ABZ apparent solubility (up to 2.226 mg.mL^{-1}) was obtained using Transcutol[®]

(40 % w/w) at a pH of 1.2 (Torrado et al, 1996). However, the use of many of these agents should be restricted whenever possible because they can be irritants to mucosa membranes.

There are some evidence in the literature that addition of cyclodextrins (CDs) into ABZ formulations could enhance ABZ oral bioavailability, particularly hydroxypropyl- β -cyclodextrin (HP- β -CD) which significantly increased ABZ absorption in comparison with a surfactant-based formulation (Ritger et al., 2004). This study conducted on healthy volunteers showed that the relative bioavailability of ABZ formulated in arachid oil-polysorbate 80 or HP- β -CD containing formulations were enhanced 4.3- and 9.7-folds, respectively, compared to commercial tablets. The interest of combining ABZ to HP- β -CD was further confirmed by Evrard et al. (2002) who showed an improvement of the oral and the effectiveness of ABZ/HP- β -CD inclusion complex against encapsulated larvae of *Trichinella spiralis* in a murine model (Casulli et al, 2006). ABZ complexed to HP- β -CD resulted in an increase of aqueous solubility of the drug, up to 3500 times at 37 °C (Bassani et al, 1996).

The use of CDs represents thus an interesting strategy for the improvement of ABZ aqueous apparent solubility. However, in spite of these numerous advantages there are no data available on the solubilisation of ABZ by using CDs other than HP- β -CD. Therefore, it was necessary to screen some pharmaceutically acceptable CDs on rationale bases in order to identify the most adapted one for optimal ABZ bioavailability. In the work to be presented here, phase solubility diagrams were used to investigate ABZ attainable solubility enhancements and to identify the best CD derivate capable of improving its aqueous solubility. Then, an in depth physico-chemical characterization including nuclear magnetic resonance (^1H NMR) coupled with two-dimensional nuclear overhauser effect (NOESY) and molecular modeling was used to investigate the mechanism of CD/ABZ complexation and to identify the chemical groups able to interact with the CD.

2. Materials and methods

2.1. Reagents

Albendazole (ABZ) ($M_w=265.33 \text{ g.mol}^{-1}$), was purchased from GlaxoSmithKline (Great Britain). Random methyl- β -cyclodextrin (Me- β -CD Rameb[®], $M_w=1331.36 \text{ g.mol}^{-1}$) was from Cyclolab (Budapest, Hungary), hydroxypropyl- β -cyclodextrin (HP- β -CD, $M_w=1375.36 \text{ g.mol}^{-1}$) was from Acros Organics (Geel, Belgium), α -cyclodextrin (α -CD, $M_w=972.85 \text{ g.mol}^{-1}$) Cavamax[®] W6 from ISP (Netherlands) and sulfobutylether- β -cyclodextrin (SBE- β -CD, $M_w=2241 \text{ g.mol}^{-1}$) Captisol[®] was a gift from CyDex Pharmaceuticals, Inc (USA). All chemicals were of analytical or reagent grade and were used without further purification. Solutions were prepared by weight using MilliQ[®] water (Millipore, Molsheim, France). Analytical grade methanol, ethanol and acetonitrile were obtained from Carlo Erba (Val de Reuil, France). Deuterium oxide (D_2O) (99.96 % D) and deuterated acetonitrile (C_2D_3N) was obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

2.2. Phase solubility diagrams and apparent solubility determinations

Phase-solubility studies were carried out according to the method described by Higuchi and Connors (1965). An excess of commercial ABZ was placed in a glass vial (20 mL) with 2 mL of solubilizing agent which was an aqueous solution of α -, HP- β -, SBE- β - or Me- β -CD at concentrations ranging from 5 % to 40 % w/w for HP- β -, SBE- β - or Me- β -CD, and from 5 % to 10 % w/w for α -CD due to its own solubility limitations at 25 °C.

Vials containing an excess of ABZ powder dispersed in a solution of CD were kept in a shaker for 5 days at 25 °C. This duration was estimated to be long enough for reaching a complexation equilibrium. Then, the samples were filtered through a 0.22 μm membrane filter (Millex, SLAP 0225, Millipore, France).

The solubility of ABZ in water was determined according to the same protocol without the presence of CDs.

2.3. HPLC Determination of ABZ concentrations.

Samples containing ABZ were analyzed by reversed phase high performance liquid chromatography (HPLC) using a symmetry C₁₈ column (5 µm, 250 x 4 mm, Waters). Chromatography was performed with a Waters 515 pump and a Waters 717Plus autosampler. UV absorbance at 235 nm was monitored with a Waters 486 absorbance detector. Data were recorded and processed on Azur Software (Datalys). The mobile phase was an isocratic mixture of acetonitrile/water (50:50 % v/v). HPLC analyses were performed at a flow rate of 0.5 mL.min⁻¹.

A calibration curve was obtained from a stock solution of ABZ/Me-β-CD complexes (250 µg.mL⁻¹) prepared according to the method described in the previous section. The concentration of ABZ should be below the solubility of the inclusion complex. The stock solution was diluted with the Me-β-CD solution. Valid concentrations for quantification were in the range 0.25-250 µg.mL⁻¹. The equation of the calibration curve was $y = 0.589x + 0.012$ ($R^2 = 0.9997$).

The calibration curve obtained with Me-β-CD was compared to the one obtained from a stock solution of ABZ dissolved in acetonitrile at a concentration of 500 µg.mL⁻¹. This calibration curve was obtained by diluting the stock solution with the mobile phase. Valid concentrations for quantification were in the range 0.25-500 µg.mL⁻¹. The equation of the calibration curve was $y = 0.5404x$ ($R^2 = 0.9999$) for ABZ in acetonitrile.

A Student's t distribution test was carried out to compare the slope and the intercept of the two previous equations. The p-values were higher than 5 %, supporting that the linear least

square lines could be considered statistically equal.

However, it was not possible to prepare sufficiently concentrated stock solutions of ABZ complexes prepared with HP- β -CD, β -CD, α -CD and SBE- β -CD due to the poor ABZ solubilisation effect obtained when using these CDs.

According to the data, there was no influence of the presence of Me- β -CD on the valid calibration curves. Consequently, the concentrations of ABZ for HP- β -CD, β -CD, α -CD and SBE- β -CD were estimated from the calibration curve obtained with acetonitrile.

2.4. Nuclear Magnetic Resonance (^1H NMR) experiments

^1H -NMR spectra were obtained by operating at 600 MHz (Bruker Avance, TXI SB 5 mm) for the free ABZ, the inclusion complex ABZ/Me- β -CD and the Me- β -CD alone. The two-dimensional nuclear Overhauser effect (NOESY) spectra of the complex were performed with a Bruker Avance, TXI Cryoprobe at 600 MHz.

The inclusion complex of ABZ and Me- β -CD (10 % w/w) for NMR analyses was prepared as described in section 2.2 by replacing water by D_2O . Free ABZ solutions were prepared by placing an excess of commercial drugs powder in a glass vial (20 mL) with 2 mL of D_2O or by dissolving ABZ in $\text{C}_2\text{D}_3\text{N}$ at a concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$. Then, the samples were filtered through a $0.22 \text{ }\mu\text{m}$ membrane filter (Millex, SLAP 0225, Millipore, France). Chemical shifts were reported in ppm (δ) downfield from tetramethylsilane (TMS) internal reference.

2.5. Molecular modeling

The molecular docking technique was used in order to gain insight into the interaction between ABZ and Me- β -CD. The Me- β -CD structure used in this work was generated from the curated coordinates of ligand QKH (structure 2QKH, Parthier et al., 2007), downloaded from

HIC-Up database (Kleywegt, 2007), by manually adding methyl groups on all free hydroxyl groups in order to obtain a fully permethylated structure (GaussView 5, Semichem Inc). All dihedral angles of the methoxy groups were then homogenized, the resulting conformations being compatible with an unhindered CD cavity. Three-dimensional coordinates of ABZ were generated using Corina version 3.44 (<http://www.molecular-networks.com/>).

Molecular docking was carried out using Gold program (Verdonk et al., 2003) and the GoldScore scoring function. The binding site was defined as a sphere with a radius of 20 Å centered on the Me-β-CD cavity. All other parameters were used with the default values.

Molecular modeling figures were generated using PyMol (<http://www.pymol.org/>). CACTVS Chemoinformatics Toolkit (<http://www.xemistry.com/>) was used for generating the PDF3D representation of the docking complex between ABZ and Me-β-CD (Supplementary Information file).

3. Results and discussion

Despite the fact that ABZ has proven to be efficient against human and animal helminthes parasites and *T. vaginalis*, its extremely low aqueous solubility represents an obstacle from a formulation point of view and also for its antiparasitic activity *in vivo*. The use of CDs represents thus an interesting strategy for the improvement of ABZ aqueous apparent solubility. The influence of the CD cavity size (α-, β-CD) and the substituents of β-CD (HP-β-, SBE-β- and Me-β-CD) on the apparent solubility of ABZ in water was investigated by phase solubility diagrams. Phase solubility diagrams are based on monitoring changes in solubility of ABZ by the addition of an excess of CDs at constant temperature. The total concentration of solubilized ABZ was plotted as a function of CD concentration. The examination of phase solubility diagrams on Fig.2 gave an overview of the process of complexation between ABZ

and the CDs based on Higuchi and Connors' classification (Higuchi and Connors., 1965). "A" type curves were obtained whatever the CD used indicating the formation of water soluble complexes on the whole range of CD concentrations investigated.

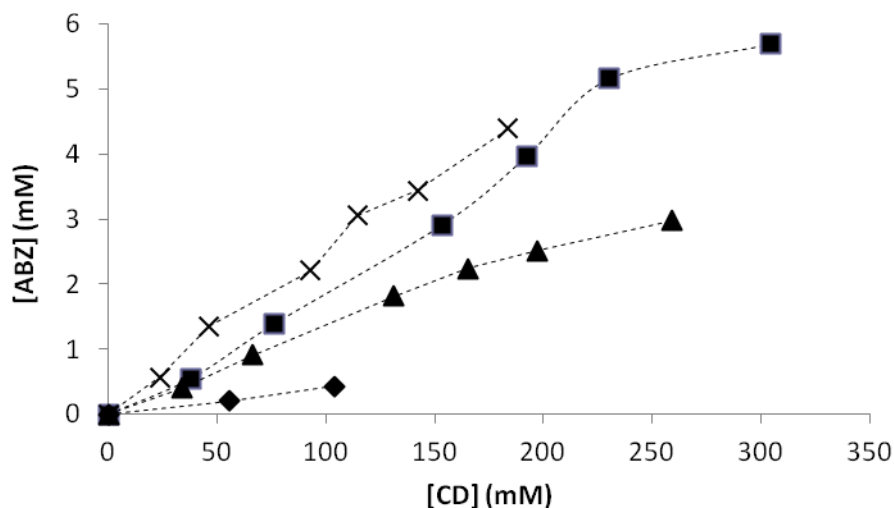


Figure 2. Experimental phase solubility diagram of ABZ for (▲) HP-β-CD and (■) Me-β-CD, (x) SBE-β-CD and (u) α-CD. (n = 3)

A linear increase in ABZ solubility as a function of CD concentrations was obtained with α- and SBE-β-CD. These CDs led to an "A_L" type diagram according to Higuchi and Connors). In this case, the apparent stability constants (also called complex formation constants or complex equilibrium constants) of ABZ with α- and SBE-β-CD was calculated according to the equation 1 (Higuchi and Connors., 1965):

$$K_{1:1} = \frac{\text{Slope}}{S_0(1 - \text{Slope})} \quad \text{Equation 1}$$

Where S_0 is the equilibrium solubility of ABZ in the absence of CD. The results indicate that the stability constants of α- and SBE-β-CD were 5510 and 32160 M⁻¹ respectively.

HP- β -CD and Me- β -CD showed a linear increase in solubility with a slightly negative deviation from linearity (type A_N) at highest CD concentrations. Higher apparent stability constant obtained with Me- β -CD (27700 M^{-1}) was much higher than the one obtained with HP- β -CD (19540 M^{-1}).

The apparent aqueous solubility of ABZ was increased with HP- β -CD (40 % w/w) up to 0.79 mg.mL^{-1} (2.79 mM). The apparent solubility enhancement factor (3970) was close to the one obtained by Bassani *et al.* (1996) using the same CD (3500). There is evidence that the two set of experiments were not conducted exactly with the same products (e.g. different batches) which could very reasonably explain the observed difference in the apparent solubility enhancement factors.

Table 1. Highest observed apparent solubilities of ABZ in different CD solutions in water. The apparent solubility enhancement factors (F) were obtained by dividing the solubility of ABZ in presence of CDs by the solubility of ABZ in water without CD*. $K_{1:1}$ is the apparent stability constant calculated from the slope of the phase solubility diagrams according to equation 1. CE is the complexation efficiency calculated according to equation 2, $[CD]/[ABZ/CD]$ is the ratio of the CD concentration allowing to reach the maximal concentration of ABZ. A 1:1 stoichiometry was assumed to calculate $K_{1:1}$. $T^\circ = 298\text{ K}$ (25 °C), $n = 3$.

CD	Maximal investigated CD concentrations		Maximal apparent solubility of ABZ		Solubility enhancement F	$K_{1:1}$ (M^{-1})	CE	$\frac{[CD]}{[ABZ/CD]}$
	% (w/w)	(mM)	($mg.mL^{-1}$)	(mM)				
None*	0	0	0.02×10^{-2}	0.75×10^{-3}	1	-	-	-
α -CD	10	103.93	0.11 ± 0.02	0.43 ± 0.05	570	5513	0.004	240
HP- β -CD	40	258.26	0.79 ± 0.04	2.97 ± 0.10	3970	19546	0.014	90
SBE- β -CD	40	183.74	1.17 ± 0.07	4.39 ± 0.08	5880	32161	0.024	40
Me- β -CD	40	304.24	1.52 ± 0.09	5.69 ± 0.09	7600	27707	0.020	50

The apparent ABZ aqueous solubility was markedly increased from $0.2\text{ }\mu\text{g/mL}^{-1}$ ($0.7\text{ }\mu\text{M}$) to 1.52 mg.mL^{-1} (5.69 mM) with Me- β -CD (40 % w/w). This apparent solubility improvement was the highest ever reported in the literature for ABZ using CDs corresponding to the apparent solubility enhancement factor of 7600. The enhancement in the apparent solubility is due to the formation of inclusion complexes between ABZ and the CD as confirmed by the ^1H -NMR (NOESY) experiments and molecular docking calculations. ^1H NMR was first carried out in order to compare the spectrum of the ABZ/Me- β -CD complex with the one of free ABZ and free Me- β -CD. However, low concentration of Me- β -CD (10 % w/w instead of 40 % w/w) was preferred for the characterization studies.

The ^1H -NMR spectrum of the inclusion complex of Me- β -CD with ABZ presented on Fig.3 showed the presence of both molecules. The chemical shifts of the peaks obtained in this region for non-complexed ABZ were compared to the ones for ABZ complexed with Me- β -CD (Table 2). The chemical groups of ABZ involved in the interaction with Me- β -CD cavity were then studied by two dimensional Nuclear Overhauser Effect measurements (^1H -NMR 2D NOESY). The chemical shifts corresponding to ABZ chemical groups able to interact with Me- β -CD were localized in the range of 3.2-4.15 ppm and the ones corresponding to Me- β -CD ranged from 3.25 to 4.1 ppm (Fig.4). The analysis of the spectra was limited to these two regions. Fig.4 showed the cross peak NOESY bands that coincided with the intermolecular interactions between the ABZ protons of the bicyclic fragment of the ABZ molecule (on C₃, C₄, C₆, C₇ and N₁₀, see figure 1 for atoms attribution) and the internal protons of the Me- β -CD cavity.

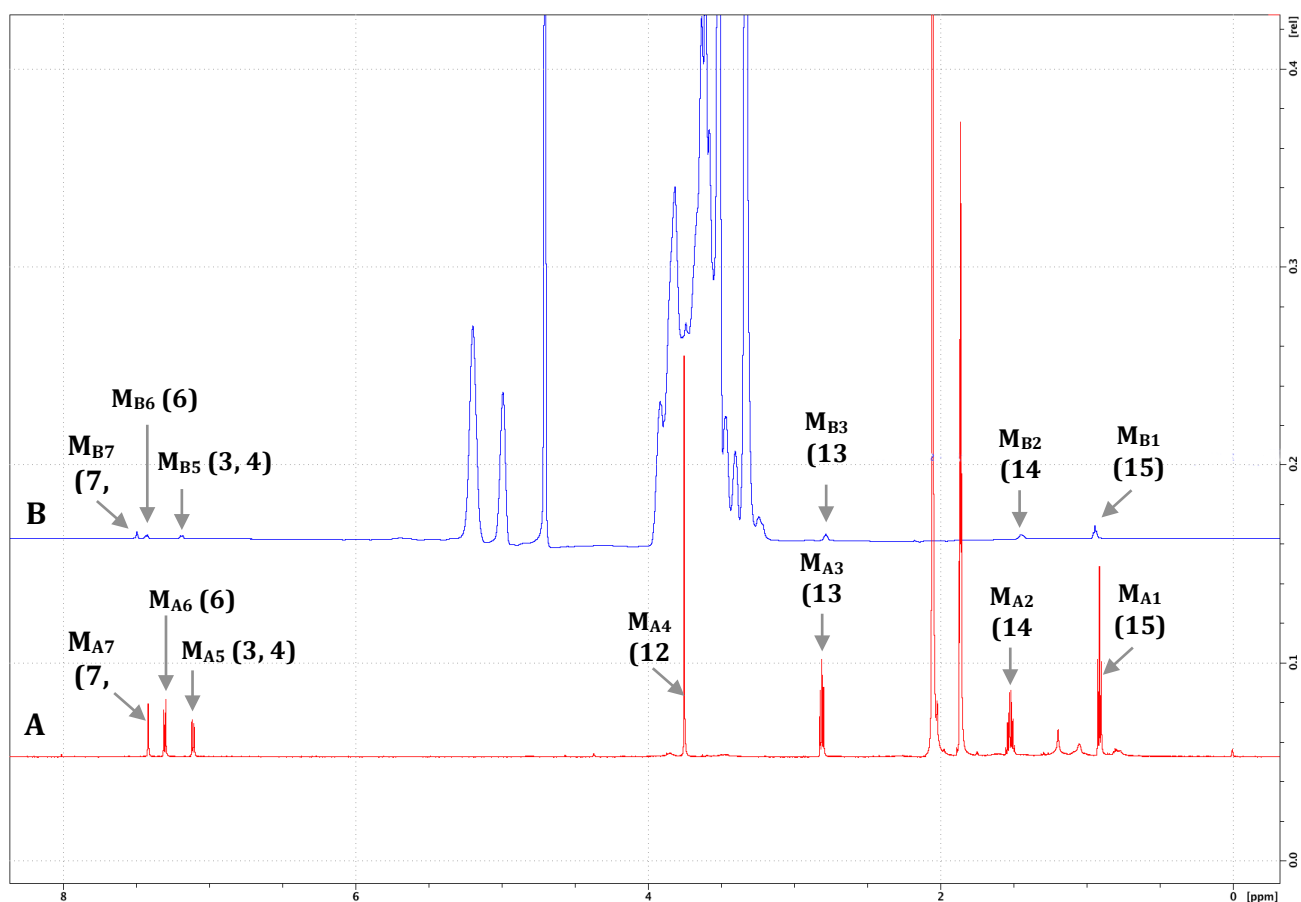


Figure 3. ^1H NMR spectra of (A) non-complexed ABZ in CD_3CN (600 MHz, NS=16, $[\text{ABZ}]=1 \text{ mg.mL}^{-1}$) and (B) ABZ/Me- β -CD inclusion complex in D_2O (600 MHz, NS=64, $[\text{ABZ}]=0.3 \text{ mg.mL}^{-1}$).

Table 2: ^1H NMR band assignments of **(A)** non-complexed ABZ in CD_3CN (600 MHz, NS=16, $[\text{ABZ}]=1\text{ mg.mL}^{-1}$) and **(B)** inclusion complex of ABZ/Me- β -CD (600 MHz, NS=64, $[\text{ABZ}]=0.3\text{ mg.mL}^{-1}$). The atom numbering is presented in Figure 1.

Atom	(A) Non-complexed ABZ		(B) Inclusion complex ABZ/Me- β -CD	
	Multiplet	Shift (ppm)	Multiplet	Shift (ppm)
15	M_{A1}	0.84 - 0.93	M_{B1}	0.90 – 0.93
14	M_{A2}	1.47 - 1.57	M_{B2}	1.40 – 1.49
13	M_{A3}	2.74 - 2.87	M_{B3}	2.74 – 2.81
12	M_{A4}	3.75	M_{B4}	Superposed to Me- β -CD
3, 4	M_{A5}	7.08 - 7.14	M_{B5}	7.14 – 7.19
6	M_{A6}	7.22 - 7.34	M_{B6}	7.36 – 7.42
7,10	M_{A7}	7.35 - 7.46	M_{B7}	7.44 – 7.48

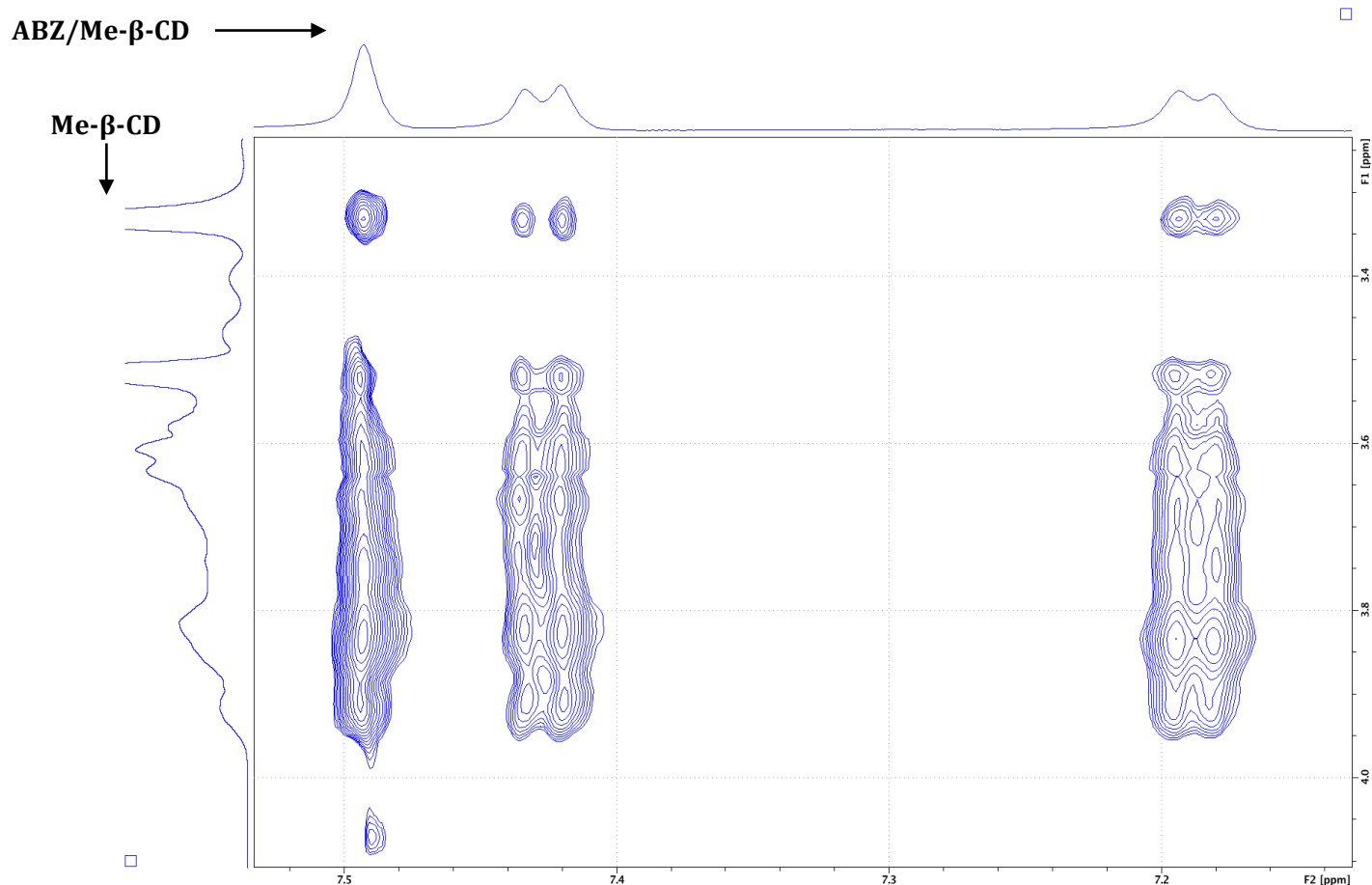


Figure 4. Two-dimensional spectrum of ABZ/Me- β -CD inclusion complex (600 MHz, D_2O , from 3.25 to 4.1 ppm) and Me- β -CD hydrogens (600 MHz, D_2O , from 7.13 to 7.53 ppm). The largest cross peak coincided with the intermolecular interactions between the hydrogen of ABZ and the internal hydrogens of the Me- β -CD cavity.

Molecular docking of ABZ within the cavity of one molecule of Me- β -CD showed in a reproducible manner a single interaction mode, with the bicyclic system in the middle of the cavity (Fig. 5 A and B). This interaction mode is typical for a 1:1 stoichiometry, supported by the almost perfect complementarity between molecular surfaces of the bicyclic system of ABZ and the Me- β -CD cavity (Fig. 5 C and D). Besides this shape complementarity, the driving force of this interaction was constituted by two hydrogen bonds between the two NH groups of ABZ and two distinct oxygen atoms from the Me- β -CD cavity (Fig. 5 E). Overall, these molecular modeling results are in very good agreement with ^1H -NMR 2D NOESY experiments confirming that the interaction of ABZ with Me- β -CD occurred through the central bicyclic fragment of ABZ.

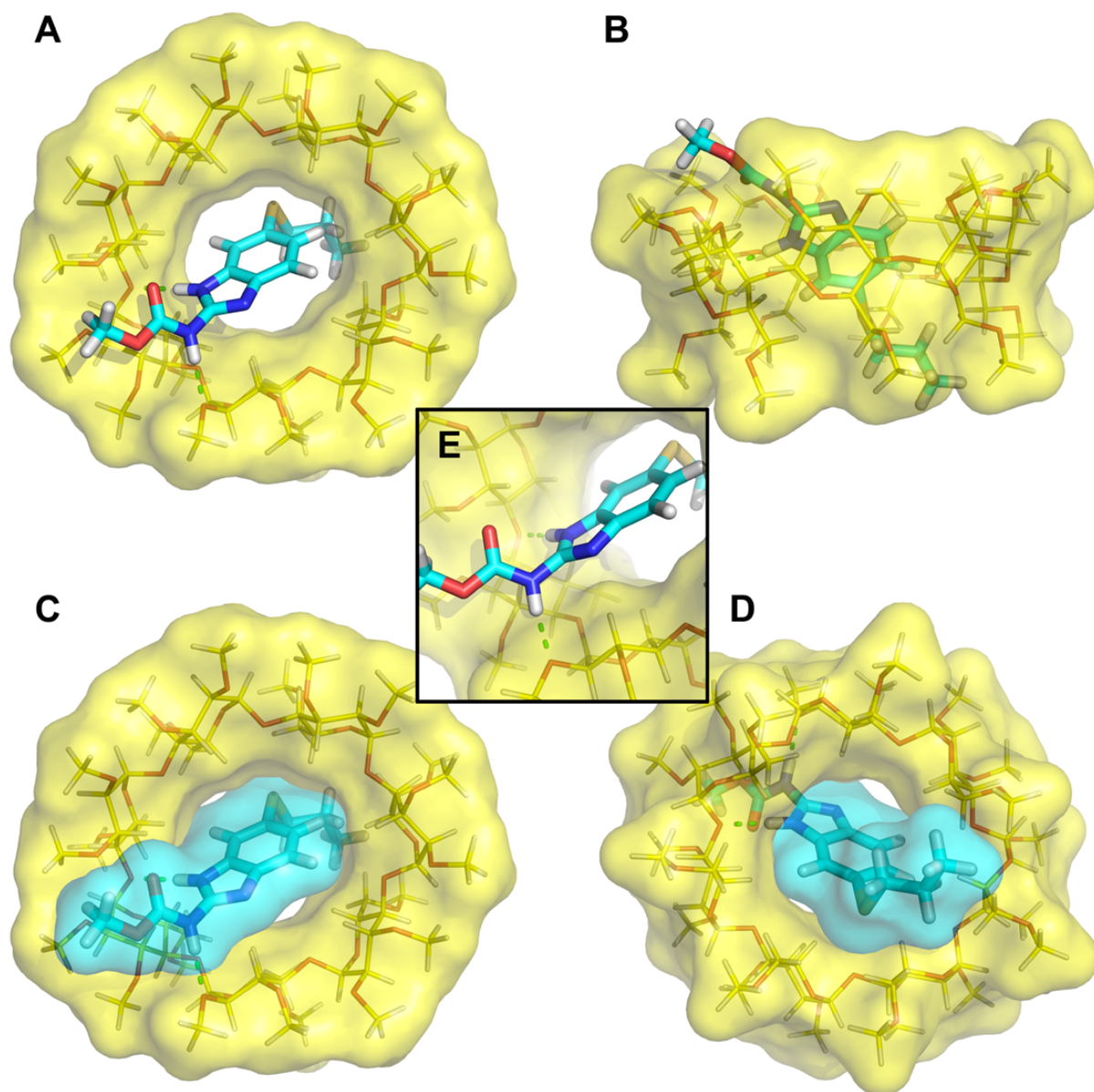


Figure 5. Representation of the complex between ABZ (cyan) and Me- β -CD (yellow) obtained by molecular docking (1:1 stoichiometry).

However, previous studies have shown that the formation of inclusion complex between the drug and CDs is not the only reason for the increase of the apparent aqueous solubility of drugs (For review see Loftsson et al., 2007). Besides inclusion complexes between ABZ and the CD, both CDs and CD complexes are known to form aggregates. These aggregates are able to increase the apparent water solubility of hydrophobic molecules through micellar-type mechanism (Loftsson et al., 2004, Magnúsdóttir et al., 2002, Loftsson et al., 2002).

Consequently, the apparent stability constants calculated from phase-solubility diagrams describe the result of all the solubilisation effects of CDs and their complexes on the ABZ solubility. Furthermore, $K_{1:1}$ is determined from S_0 which is inaccurate for compounds with $S_0 < 0.1$ mM (Loftsson et al., 2005) leading to erroneous $K_{1:1}$ values.

Another method for the determination of the efficiency of the solubilisation of a CD is to calculate the complexation efficiency (CE) according to Equation 2. The CE reflects the concentration ratio between CD in a complex and free CD.

$$CE = \frac{\text{Slope}}{1 - \text{Slope}} = \frac{[ABZ/CD]}{[CD]} \quad \text{Equation 2}$$

Where $[ABZ/CD]$ is the concentration of dissolved complex, $[CD]$ the concentration of dissolved free cyclodextrin.

Of the CDs tested, SBE- β -CD and Me- β -CD have the highest CE which were about 0.024 and 0.020 respectively. This means that only about one out of every 40 SBE- β -CD molecules in solution are forming a water-soluble complex with ABZ, while one out of every 50 Me- β -CD molecules in solution are forming a water-soluble complex with ABZ. The CE was the lowest one for α -CD demonstrating the low efficiency of encapsulation for this CD. These data are consistent with the size-fit concept, which predicts the highest complex stabilities for the best size-matched host-guest pairs. Indeed, ABZ molecule interacts more favorably with β -type CDs because the 6.0–6.5 Å° cavity diameter for these CDs is able to accommodate aromatic groups found in ABZ molecule while α -CD cavity (4.7-5.3 Å°) is only able to accommodate alkyl chains (Sanemasa et al., 1990; Rekharsky et al., 1994).

5. Conclusion

Among the four CDs compared in this study for their ability to solubilize ABZ, Me- β -CD showed the highest potential to increase the apparent aqueous solubility, with the highest solubility enhancement factor ever reported in the literature for ABZ by using conventional and pharmaceutically acceptable CDs. The combination of solubility experiments to ^1H NMR-NOESY and molecular docking calculations showed that the central bicyclic fragment from ABZ was able to interact with the Me- β -CD accordingly to a 1:1 stoichiometry. This formulation will be then studied for its biological activity on infectious models for which ABZ is used in therapy.

6. Acknowledgments

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Chapitre 4: Thermosensitive hydrogel containing
metronidazole active against *Trichomonas vaginalis*:
formulation, *in vitro* release and *ex vivo* penetration
through pig vaginal mucosa

Thermosensitive hydrogel containing metronidazole
active against *Trichomonas vaginalis*: formulation, *in vitro* release and *ex vivo* penetration through pig vaginal
mucosa

Bénédicte Pradines^{a,b}, Vanessa Lievin-LeMoal^b, Christian Bories^b, Christine Vauthier^a, Gilles Ponchel^a, Philippe M. Loiseau^b, Kawthar Bouchemal^a

^a Institut Galien Paris Sud, UMR CNRS 8612, Faculty of Pharmacy, Univ Paris-Sud, France

^b Antiparasitic Chemotherapy, UMR CNRS 8076, Faculty of Pharmacy, Univ Paris-Sud,
France

Abstract

The objective of this work is to investigate the potential of thermosensitive hydrogels to increase the local efficacy of vaginally-administrated metronidazole (MTZ) for the treatment of *Trichomonas vaginalis*-related infections. First, the apparent aqueous solubility of MTZ was significantly increased from 53.7 mM to 200.1 mM using different cyclodextrines (CDs) but CDs presented toxicity against HeLa cells. The adjusted formulation was thus composed of hydrogels were composed of pluronic[®] F127 (20 wt%), chitosan (1 wt%) as mucoadhesive polymer and MTZ (0.70 wt%). This hydrogel formulation without CDs did not exhibit *in vitro* toxicity against HeLa cells and increased the release time of MTZ in comparison with formulations without hydrogel. Hydrogel limited MTZ passage through vaginal mucosa therefore promoting local administration. This formulation exhibited an interesting *in vitro* trichomonacidal activity with an IC₂₀ about 0.35 µg.mL⁻¹ of MTZ.

Keywords : Pluronic[®] F127, thermosensitive, hydrogel, *Trichomonas vaginalis*, HeLa cells, pig vaginal mucosa.

1. Introduction

Trichomoniasis is an extremely common cosmopolitan genitourinary Sexually Transmitted Infection (STI) in humans caused by the parasite *Trichomonas vaginalis* (*T. vaginalis*). This disease has important medical, social, and economical implications. The World Health Organization (WHO) estimates that the total number of new cases of curable STIs in 2008 in adults between the ages of 15 and 49 was about 498.9 million with 276.4 million cases resulting from *T. vaginalis*. In 2008, the number of new cases of infections with *T. vaginalis* was estimated to be 11.2% higher than for 2005 (276.4 million versus 248.5 million) (Rowley et al. 2012). Reports have implicated *T. vaginalis* in the post-surgical infections of the upper reproductive tract, reversible infertility, premature rupture of the placental membranes, premature labor, low-birth-weight infants, neonatal morbidity and mortality (Cotch 1990; Cotch et al. 1997; Hardy et al. 1984; Minkoff et al. 1984).

Currently, the nitroimidazole chemical family is the only class of drugs useful as oral or parenteral therapy against trichomoniasis. However, nitroimidazole-resistance and toxicity justify alternative approaches (Schmid et al. 2001; Jane R. Schwebke and Barrientes 2006). Currently, topically-applied treatments should be limited to adjunctive therapy or particular cases of allergy or resistance. Nevertheless, some data have shown that research is needed to define the optimal dose of the active product in vaginal formulations and that further refinement is required before the process can be put into practice, treatment of trichomoniasis by the vaginal route being a real alternative to systemic treatment.

In this purpose, the present work aimed to combine the effect of hydrogels containing thiolated chitosan and metronidazole (MTZ) for the design of new vaginally-applied microbicide formulations for the prevention and the treatment of trichomoniasis. Thiolated

chitosan was used because we have already shown its capacity to improve mucoadhesion (Petit et al. 2012).

The thermosensitivity of the hydrogel at the body temperature is a desirable characteristic, which is likely to make easier the application of the formulations in the vaginal cavity. Once gelified, due to temperature increase, this hydrogel would develop mucoadhesive characteristics facilitating thus the presence MTZ in the vaginal cavity and can help to maintain high local drug concentrations at the vaginal epithelium surface. Polymeric mucoadhesive hydrogels will be selected as a basis for creating an intimate and prolonged contact at the site of administration. An improved patient compliance is expected because of a possible reduction of the frequency of administration as well as the use of lower doses of drugs.

Obviously, conventional dosage forms such as solutions, ointments or creams cannot satisfy simultaneously these objectives, mostly because they are typically removed from the mucosal tissue within seconds to a few hours, thereby strongly limiting the duration of local formulations.

In this context, the strategy we envisioned in the present study is to increase the apparent aqueous solubility of MTZ using cyclodextrins (CDs). Then, an *in vitro* studies of the release of MTZ, an *ex vivo* study of the mucosal passage of MTZ, an *in vitro* cell toxicity and trichomonacidal activity of hydrogel containing metronidazole were performed.

2. Materials

Pluronic[®] F127 (Poloxamer P407, EO₉₈PO₆₉EO₉₈) and pluronic[®] F68 (Poloxamer P188, EO₈₀PO₂₇EO₈₀) copolymers of pharmaceutical grade were from BASF ChemTrade GmbH (Ludwigshafen, Germany). Hydroxypropyl- β -cyclodextrin (HP- β -CD, $M_w=1375 \text{ g.mol}^{-1}$) was from Acros Organics (Geel, Belgium), Random methyl- β -cyclodextrin (Me- β -CD Rameb[®], $M_w=1331.36 \text{ g.mol}^{-1}$) was from Cyclolab (Budapest, Hungary) and Rameb methyl- β -cyclodextrin Me- β -CD (Crismeb[®], $M_w=1191 \text{ g.mol}^{-1}$) were from Roquette (Lestrem, France). Solutions were prepared by weight using MilliQ[®] water (Millipore, Molsheim, France). Analytical grade methanol was from Carlo Erba (Val de Reuil, France).

For cell culture: Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and fetal calf serum were from Invitrogen, Saint Aubin, France. Amino-Acid Non Essential 1 % (AANE 100X, Gibco, France). Trypsin and ethylenediamine tetraacetic acid EDTA were from Sigma-Aldrich (Saint-Quentin Fallavier, France), culture plates (TPP, Dutscher, France). Trypan blue was from VWR (France). Glucose and nonessential amino acids were from Life Technologies (Cergy, France).

All other reagents used for the preparation of SVF and phosphate buffer saline (PBS) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Commercial chitosan (20 kDa, DDA 80–85 %) from Amicogen (Seoul, South Korea) was thiolated. The resulting polymer is chitosan-4-thiol-butylamidine named Chito20-TBA. Bactotryptone and Yeast extract were from BD Difco. Maltose, L-cysteine hydrochloride and L-ascorbic acid were from Sigma-Aldrich (Saint-Quentin Fallavier, France). All other reagents were supplied by Sigma-Aldrich (Saint-Quentin Fallavier, France) and were of analytical grade.

T. vaginalis strain CMP (Châtenay Malabry Parasitology) was isolated from a woman suffering from STD in 1987 and stored as stabilized in liquid nitrogen containing 6 % dimethyl sulphoxide (DMSO) as cryoprotectant. This strain was metronidazole-sensitive.

3. Methods

3.1 Preparation of the hydrogels

First a solution containing 1 % of chitosan and thiolated chitosan (Chito20/Chito20-TBA) at different percentages (75/25 % and 100/0 %) and/or 7 mg.mL⁻¹ of MTZ was prepared in a lactate buffer (pH 4.5; 50 mM). The formulations containing 25 % of chitosan 20-TBA were denoted (Chito/Chito-TBA) and the formulation containing 100 % of chitosan was simply named chitosan.

Hydrogels were prepared according to the so-called “cold method” (Aka-Any-Grah et al. 2010). Pluronic[®] F127 hydrogel (20 wt%) was obtained by gradually adding under stirring the F127 powder to the previous solutions maintained at 4 °C. The drug-free formulations were denoted: F127/Chitosan, F127/(Chito/Chito-TBA). MTZ-containing formulations were denoted: F127/Chitosan-MTZ, F127/(Chito/Chito-TBA)-MTZ.

3.2. Phase solubility diagrams and apparent aqueous MTZ solubility determinations

Phase-solubility studies were carried out according to the method described by Higuchi and Connors (1965) (Higuchi and Connors 1965). An excess of commercial MTZ was placed in a glass vial (20 mL) with 2 mL of solubilizing agent which was an aqueous solution of CDs at increasing concentrations ranging from 5 % to 40 % w/w for Me-β-CD Rameb, Me-β-CD crismeb or for HP-β-CD and from 0.5 to 1.5 % w/w for β-CD due to its own water solubility

limitations at 25 °C.

Vials containing an excess of drug powder dispersed in a solution of CD were kept in a shaker during 5 days at 25 °C. This duration was estimated to be long enough for reaching the complexation equilibrium. Then, the samples were filtered through a 0.22 µm membrane filter (Millex, SLAP 0225, Millipore, France).

The solubility of MTZ in water was determined according to the same protocol without the presence of CDs.

The MTZ concentration was determined by HPLC.

3.3 *In vitro* study to evaluate the toxicity of the formulations on HeLa cells

Human cervix HeLa cells were grown in DMEM with L-glutamine (Invitrogen, Fisher, France) supplemented with 10 % heat-inactivated fetal calf serum (Invitrogen, Fisher, France) and 1 % Amino-Acid Non-Essential (AANE 100X, Gibco, Invitrogen, Fisher, France) at 37 °C in an atmosphere containing 5 % CO₂. Cell lines were harvested from the flask using trypsin (0.5 mg.mL) or EDTA (0.2 mg.mL⁻¹), washed once with culture medium, and seeded into culture plates (TPP, Dutscher, France) at the appropriate cell densities (150,000 cell/well), before being incubated at 37 °C in an atmosphere containing 5 % CO₂ before experiments began.

For maintenance purposes, cells were passaged weekly using 0.02 % trypsin in Ca²⁺Mg²⁺-free PBS containing 3 mM EDTA. Experiments and cell maintenance were carried out at 37 °C in a 10 % CO₂/90 % air atmosphere. The culture medium was changed daily.

The evaluation of the toxicity of the formulations was achieved by exposing HeLa cells to formulation containing 1 % of chitosan20/chitosan20-TBA (75/25) or (100/0) and MTZ

jellified or no with pluronic[®] F127 (20 wt%) during 24 h. The results were compared to formulations without chitosan and MTZ (pluronic[®] F127 20 wt%) and to lactate buffer.

HeLa cells were seeded in a 24-well culture plate at 1.5×10^5 cells and incubated for 24 h at 37 °C in 5 % CO₂ prior to the test. Prior to contact, the confluent HeLa cells were washed twice with PBS, and then put in contact with formulation at appropriate concentration. The plates were incubated for 24 h at 37 °C in a humidified atmosphere containing 5 % CO₂. At the end of the contact period, the cells were washed two times with sterile PBS to remove non-adhering gels and then processed for analysis.

The quantification of viable HeLa cells was conducted with Trypan blue numeration (VWR, France) in Malassez cell.

For statistical analysis t student's t-test was applied ($p = 0.05$).

3.4 *In vitro* and *ex vivo* study using Franz diffusion cell

3.4.1 In vitro release experiment

Preparation of Simulated Vaginal Fluid (SVF)

SVF was prepared as previously described (Owen and Katz 1999). NaCl (3.51 g), KOH (1.4 g), Ca(OH)₂ (0.22 g), bovine serum albumin (0.018 g), lactic acid (2.00 g), acetic acid (1.00 g), glycerol (0.16 g), urea (0.4 g) and glucose (5.00 g) were added to 900 mL of distilled water contained in a beaker and stirred mechanically until complete dissolution. The pH of the mixture was then adjusted to 4.5 using HCl 0.1N, and the final volume was adjusted to 1 L. The pH was fixed at 4.5 within the range of the normal, premenopausal vaginal pH.

Release experiment

Franz diffusion cells were used to investigate the effect of the formulation on the release behavior of MTZ. A plastic membrane (PVDF, 0.45 μm pore size, Millipore, MA) was placed in Franz diffusion cell and the receptor compartment was filled with SVF at pH 4.5.

The investigated formulations are: MTZ in solution, F127/Chitosan/MTZ or Chitosan/MTZ solution. SVF was used as negative control.

Then, 1 mL of each formulation were placed on the filter and Franz diffusion cell were closed and placed in a thermostated bath at 37 °C.

At pre-set time intervals (0, 0.25, 0.5, 1.5, 2, 3, 6, 18 and 24 h), aliquots of 1 mL were recovered from the receptor compartment and replaced with the same volume of fresh SVF pre-equilibrated at the experimental temperature conditions (37 °C). Then, the MTZ concentration was determined by high-performance liquid chromatography (HPLC).

3.4.2 Ex vivo study by Franz cell

Preparation of pig vaginal mucosa

Experiments were carried out on female pigs (INRA Jouy en Josas, France) weighing between 60 and 63 kg in average. The animals were fasted for 24 h but had free access to tap water. All experiments on animals adhered to the Communities Commission Directive (DE/86/609/CEE) and were performed in conformance with the French Ministry of Agriculture Permission No. 78-16. Pigs were sacrificed by intravenous injection (20 mL) of overdosed sodium phenobarbital (Dolethal, Vetoquinol Laboratory, Lure, France), and the vaginal mucosa was taken over a 10 cm length. The mucosa was placed in SVF and stored at -20 °C immediately after sacrifice of the animal and kept at this temperature until use. It has been shown that porcine mucosa can be frozen during storage without affecting the mucus

layer (Squier et al. 2008). Samples were cut in 1 cm square pieces by means of surgical scissors to obtain intact vaginal tissue and defrosted before experiments at ambient temperature in the presence of SVF.

Study of MTZ mucosal passage

Franz cell were used to determine the permeability of vaginal tissue. Mucosa was placed in Franz cell in which is placed in donor medium SVF at pH 4.5.

Then, 1 mL of SVF, F127/Chitosan, F127/Chitosan-TBA, Chitosan/MTZ, Chitosan-TBA/MTZ, F127/Chitosan/MTZ or F127/Chitosan-TBA/MTZ were placed on the mucosa and Franz cell were closed and placed in a thermostated bath at 37 °C.

At pre-set time intervals (0, 0.5, 1, 2, 3, 4, 5 and 6 h), aliquots of 1 mL were recovered from the receptor compartment and replaced with the same volume of fresh SVF pre-equilibrated at the experimental temperature conditions (37 °C).

After 6 h the mucosa was placed in water overnight and MTZ concentration

Then, MTZ concentration was determined by high-performance liquid chromatography (HPLC).

3.5 Determination of MTZ concentration by high-performance liquid chromatography (HPLC)

The samples containing MTZ were analyzed by reversed phase high performance liquid chromatography (HPLC) using a symmetry C18 column (5 µm, 250 x 4 mm, Waters). Chromatography was performed with a Binary Waters 1525 pump and a Waters 717Plus autosampler. UV absorbance at 230 nm was monitored with a Waters UV 2996 absorbance

detector. Data were recorded and processed on Empower Software. The mobile phase was an isocratic mixture of methanol/water (75:25 % v/v). HPLC analyses were performed at a flow rate of 0.5 mL.min⁻¹.

For *in vitro* experiments the calibration curve was obtained from a stock solution of MTZ in lactate buffer pH 4.5 at 50 mM. The stock solution was diluted with SVF. Valid concentrations for quantification were in the range 0.25-500 µg.mL⁻¹. The equation of the calibration curve was $y = 115508x + 15488$ ($R^2 = 0.9994$).

For the determination of aqueous solubility the calibration curve was obtained from a stock solution of Me-β-CD/MTZ and HP-β-CD/MTZ complex (500 µg.mL⁻¹) prepared according to the method described in the previous section. The concentration of MTZ should be below the solubility of the inclusion complex. The stock solution was diluted respectively with the Me-β-CD and HP-β-CD solutions. Valid concentrations for quantification were in the range 0.25-500 µg.mL⁻¹. The equations of the calibration curves obtained with Me-β-CD and HP-β-CD were respectively $y = 147036x + 14484$ ($R^2 = 0.99996$) and $y = 111128x + 15185.1$ ($R^2 = 0.99953$).

The calibration curve was compared to the one obtained from a stock solution of MTZ dissolved in water at a concentration of 500 µg.mL⁻¹. This calibration curve was obtained by diluting the stock solution with the mobile phase. Valid concentrations for quantification were in the range 0.25-500 µg.mL⁻¹. The equation of the calibration curve was $y = 111128x + 9185.1$ ($R^2 = 0.99953$) for MTZ in water.

A Student's t distribution test was carried out to compare the slope and the intercept of the four previous equations. The p-values were higher than 5 %, supporting that the linear least square lines could be considered statistically equal.

However, it was not possible to prepare sufficiently concentrated stock solutions of CTZ complexes prepared with HP- β -CD and β -CD due to the poor CTZ solubilisation effect obtained using these CDs.

According to the data, there was no influence of the presence of Me- β -CD, HP- β -CD or SVF on the valid calibration curves. Consequently, the concentrations of MTZ were estimated from the calibration curve obtained with water.

3.6. Evaluation of the trichomonacidal activity of the formulations

T. vaginalis was cultured axenically at 35 °C in trypticase-yeast extract-maltose (TYM) (Diamond 1957) supplemented with 10 % horse serum (Gibco, France) and subcultured every 2 days. (Camuzat-Dedenis et al. 2001).

Culture tubes with fresh TYM medium enriched with filtered horse serum 10 % alone or with (in triplicate) the tested compound, were inoculated with 2×10^5 protozoa in 3 mL. The tubes were cultivated in conditions for 24 h at 35 °C and the number of parasites mL^{-1} in each tube determined with an haemocytometer (Kova[®] Glasstic[®] Slide 10, Hycor Biomedical, United States). The experiments were run three times.

The results were estimated as the percentage of growth inhibition compared with only excipient treated controls and plotted as profit value as a function of drug concentration ($n = 3$).

For statistical analysis, a t student's t-test was applied ($p = 0.05$).

3.7. Coculture HeLa/*T. vaginalis*

HeLa cells and *T. vaginalis* parasites were grown as explained previously.

HeLa cells were seeded in a 24-well culture plate at 1.5×10^5 cells and incubated for 24 h at 37 °C in 5 % CO₂ prior to the test. Prior to contact, the confluent HeLa cells were washed twice with PBS, and then put in contact with 200 000 of *T vaginalis*.

Then, the coculture was put in contact with the formulation at appropriate concentration.

The plates were incubated for 24 h at 37 °C in a humidified atmosphere containing 5 % CO₂. At the end of the contact period, the cells were washed two times with sterile PBS to remove non-adhering *T vaginalis* and then processed for analysis. HeLa and *T vaginalis* were harvested from the flask using trypsin (0.5 mg.mL^{-1}).

The number of adherents and mobiles parasites per milliliter in each plate determined with an haemocytometer (Kova[®] Glasstic[®] Slide 10, Hycor Biomedical , United States). The experiments were run three times.

The results were estimated as the percentage of growth inhibition compared with only excipient treated controls (n = 3). For statistical analysis t student's t-test was applied ($p = 0.05$).

For statistical analysis, a t student's t-test was applied ($p = 0.05$).

4. Results and discussion

The aim of the present work was to investigate the possibility optimize vaginal microbicide formulations by using hydrogels containing MTZ. The first issue consisted in evaluate the *in vitro* toxicity of the formulations against HeLa cells. *In vitro* release and *ex vivo* study of mucosal passage were then studied using Franz cells. Finally, the *in vitro* efficacy of the formulation was studied against *T. vaginalis*.

4.1 Phase solubility diagrams and apparent solubility determinations

Despite the fact that MTZ has proven to be efficient against *T. vaginalis*, its biodisponibility represents an obstacle from a formulation point of view. The main objective of the present work is to encapsulate MTZ in CDs to increase the release time of the molecule.

The use of CDs represents thus an interesting strategy for the improvement of MTZ biodisponibility and aqueous solubility. The influence of the CD substituents of β -CD (β -CD, HP- β -CD, Me- β -CD Rameb and Me- β -CD crismeb) on the apparent solubility of MTZ in water was investigated by phase solubility diagrams. The examination of phase solubility diagrams on Figure 1 gave an overview of the process of complexation between MBZ and the CDs based on Higuchi and Connors' classification (Higuchi and Connors 1965). "A" type curves were obtained whatever the CD used indicating the formation of water soluble complexes on the whole range of CD concentrations investigated. Type "A" diagrams are represented by a linear increase in solubility " A_L ". A linear increase in MTZ solubility as a function of CD concentration was obtained with the four type of CDs (type " A_L "). The A_L diagram is observed if all inclusion complexes are of the first order in CD.

The apparent solubility enhancement of MTZ was low using β -CD and Me- β -CD Crismeb (respectively up to 9.55 and 20.01 mg.mL⁻¹), while HP- β -CD 40 % w/w allowed to reach a MTZ concentration of 30.14 mg.mL⁻¹. The highest increase of apparent MTZ solubility was obtained using Me- β -CD Rameb 40 % w/w (34.26 mg.mL⁻¹). This was the highest increase of MTZ apparent solubility ever reported in the literature using conventional CDs.

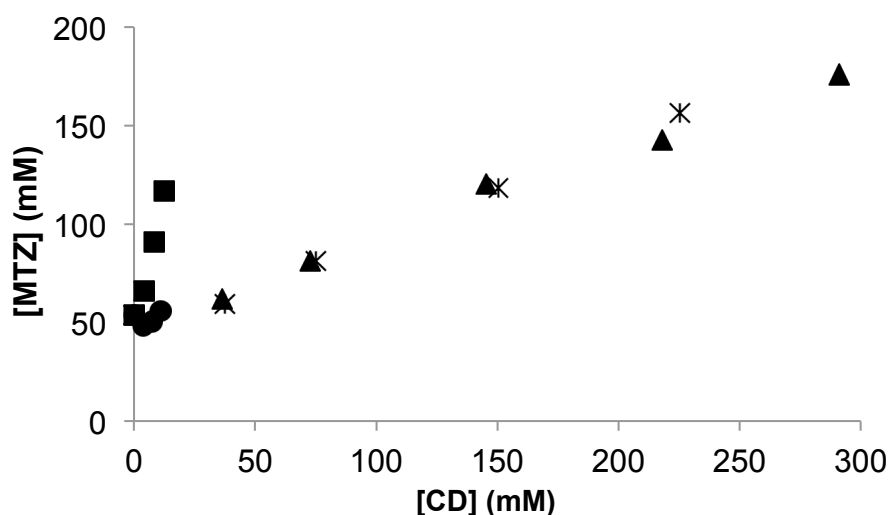


Figure 1. Experimental phase solubility diagram of MTZ for different CDs (n = 3). (●) β -CD, (*) HP- β -CD and (▲) Me- β -CD (Ramed) (■)Me- β -CD (Crismeb).

Table 1. Highest observed apparent solubilities of MTZ in different CD solutions in water. The apparent solubility enhancement factors (F) were obtained by dividing the solubility of MTZ in presence of CDs by the solubility of MTZ in water without CDs*. T° = 298 K (25 °C), n = 3.

(25 °C), n = 3.	CD	CD	Maximal apparent solubility of MTZ		F	
		% (w/w)	(mM)	(mg.mL ⁻¹)		(mM)
	None*	0	0	9.19 ± 0.03	54.72 ± 0.02	1
	β-CD	1.5	10.90	9.55 ± 0.06	55.81 ± 0.05	1.02
	HP-β-CD	40	290.83	30.14 ± 0.02	176.11 ± 0.01	3.22
	Me-β-CD Crismeb	15	12.59	20.01 ± 0.01	116.91 ± 0.01	2.14
	Me-β-CD Rameb	40	300.44	34.26 ± 0.02	200.19 ± 0.01	3.65

4.2. *In vitro* study to evaluate the toxicity of the formulations on HeLa cells

The toxicity of the formulations was studied against HeLa cells, which are human cervical carcinoma cells (Fig. 2). After 24 h of incubation period, the formulation without cyclodextrins did not present any toxicity against HeLa cells. However, all the HeLa cells in contact with Me- β -CD were lysed. So, the Me- β -CD used in our conditions were very toxic against the cells.

The toxicity of the other formulations which did not contain CDs was studied against HeLa cells (Fig 3.). After a 24 h incubation period, the F127 hydrogels have no significant toxicity against HeLa cells. Only the formulation containing MTZ and Chitosan-TBA present a significant toxicity. So, the chitosan-TBA will not be used in the final formulation.

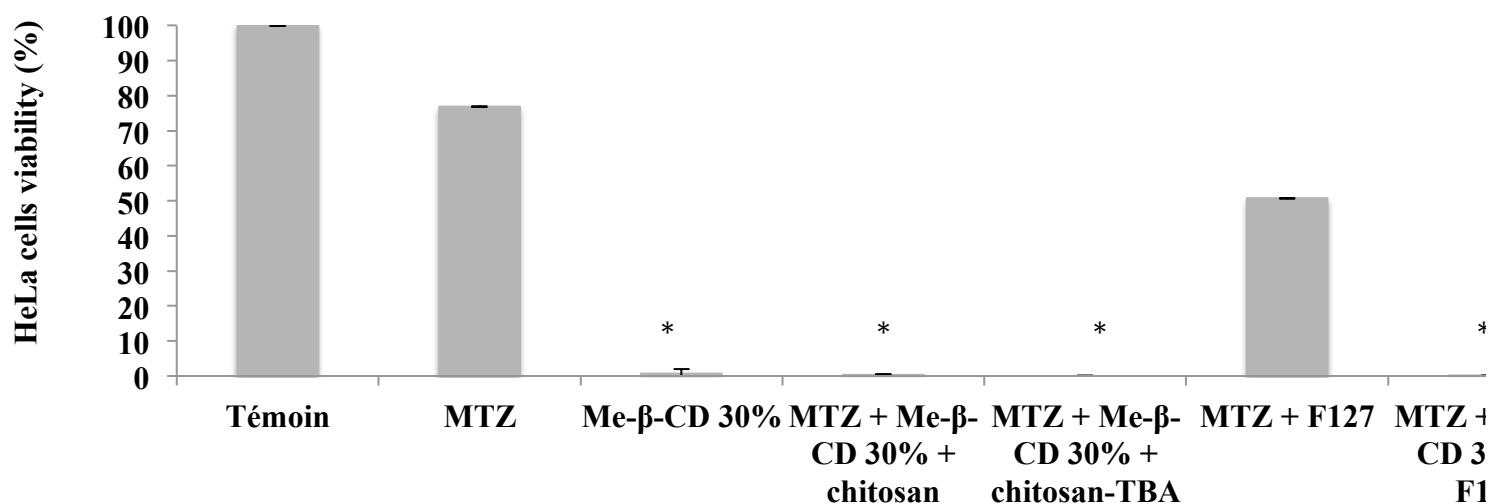


Figure 2. Effect of Me- β -CD on HeLa cells viability after 24 h of contact with different formulations. Culture medium (Control) was used as negative control. MTZ was at a concentration of 7 mg/ml, chitosan was at 1% (w/w) and pluronic[®] F127 was at 20% (w/w). Data are the mean of three determinations \pm sd. (* significant versus control $p < 0.05$)

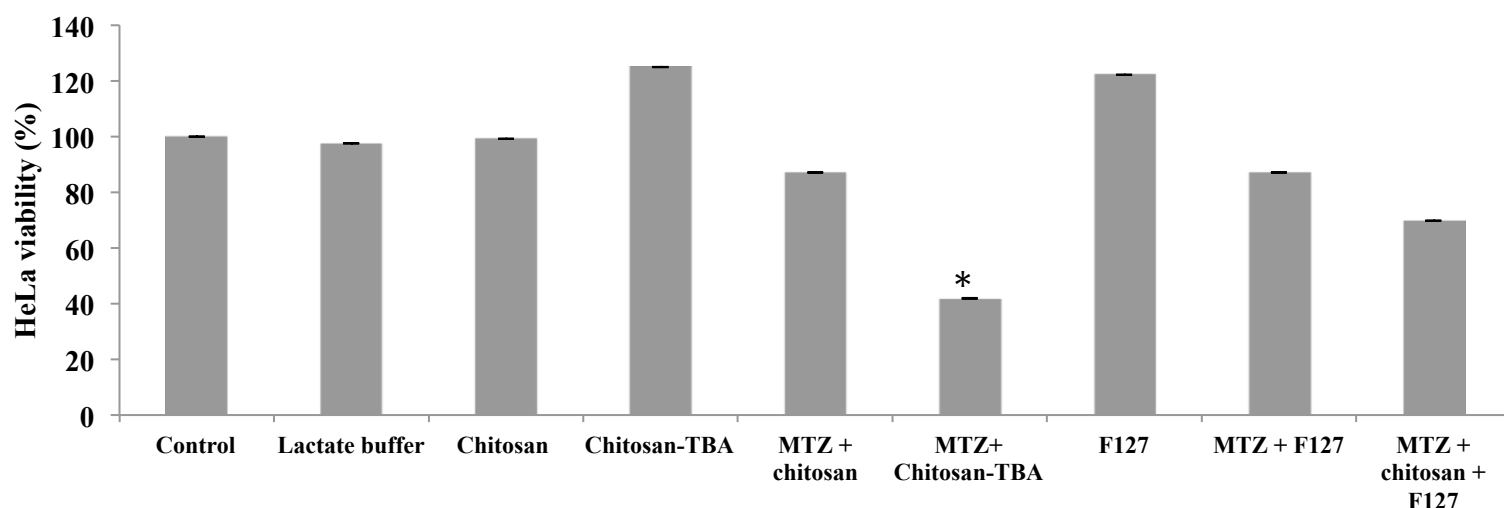


Figure 3. HeLa cell viability after 24 h of contact with different formulations. The results were compared to lactate buffer (pH 4.5 50 mM). Culture medium (Control) was used as negative control. MTZ was at a concentration of 7 mg/ml, chitosan was at 1% (w/w) and pluronic® F127 was at 20% (w/w). Data are the mean of three determinations \pm sd. (* significant versus control $p < 0.05$)

4.3 *In vitro* and *ex vivo* study using Franz cell

4.3.1 *In vitro* release experiment

The release time of MTZ of liquid and jellified formulations was studied with a plastic membrane placed on Franz cells (Fig. 4). With liquid formulation 100 % of MTZ was immediately released in receptor compartment. However only 68 % of MTZ containing in hydrogels was liberated after 24 h. Hydrogel formulation increased significantly the release time of MTZ.

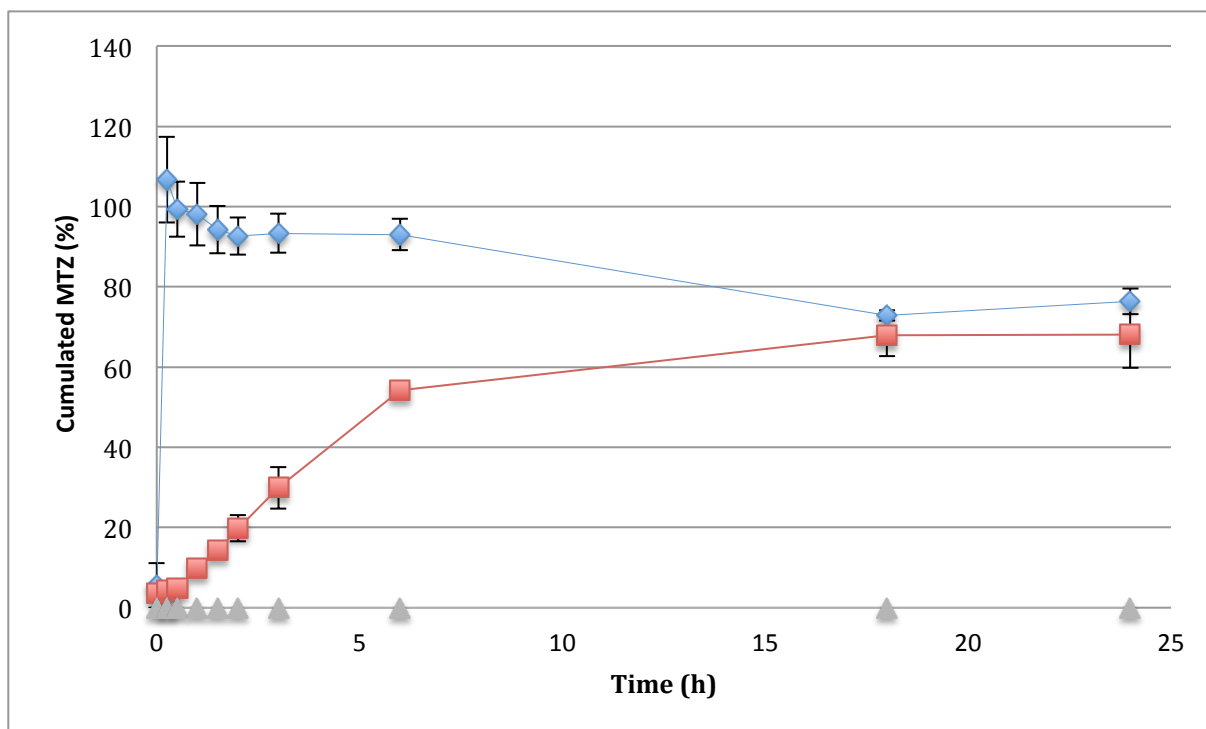


Figure 4. MTZ liberation (in %) in function of time for Chitosan/MTZ in solution (♦) and jellified formulation F127/Chitosan/MTZ (■). SVF (▲) was used as negative control. MTZ was at a concentration of 7 mg/ml, chitosan was at 1% (w/w) and pluronic® F127 was at 20% (w/w). Data are the mean of three determinations \pm sd. Low values of scale bars are included in the graphics can not be seen.

4.3.2. *Ex vivo* study by Franz cell

Franz cell were used to determine the permeability of vaginal tissue. Pig vaginal mucosa was placed in Franz cell and MTZ concentration was measured in receptor compartment filled with SVF (Fig 5.). With liquid formulations more than 10 % of MTZ crossed vaginal mucosa. However with hydrogel only less that 3.5 % of MTZ crossed vaginal mucosa. Moreover in presence of chitosan-TBA MTZ passage is more important whatever the formulation liquid or gel. Results at 12 h were in accordance with the precedents same results even if the entire mucosa is implicated. Hydrogel promote the local activity of MTZ and limit it passage trough vaginal mucosa.

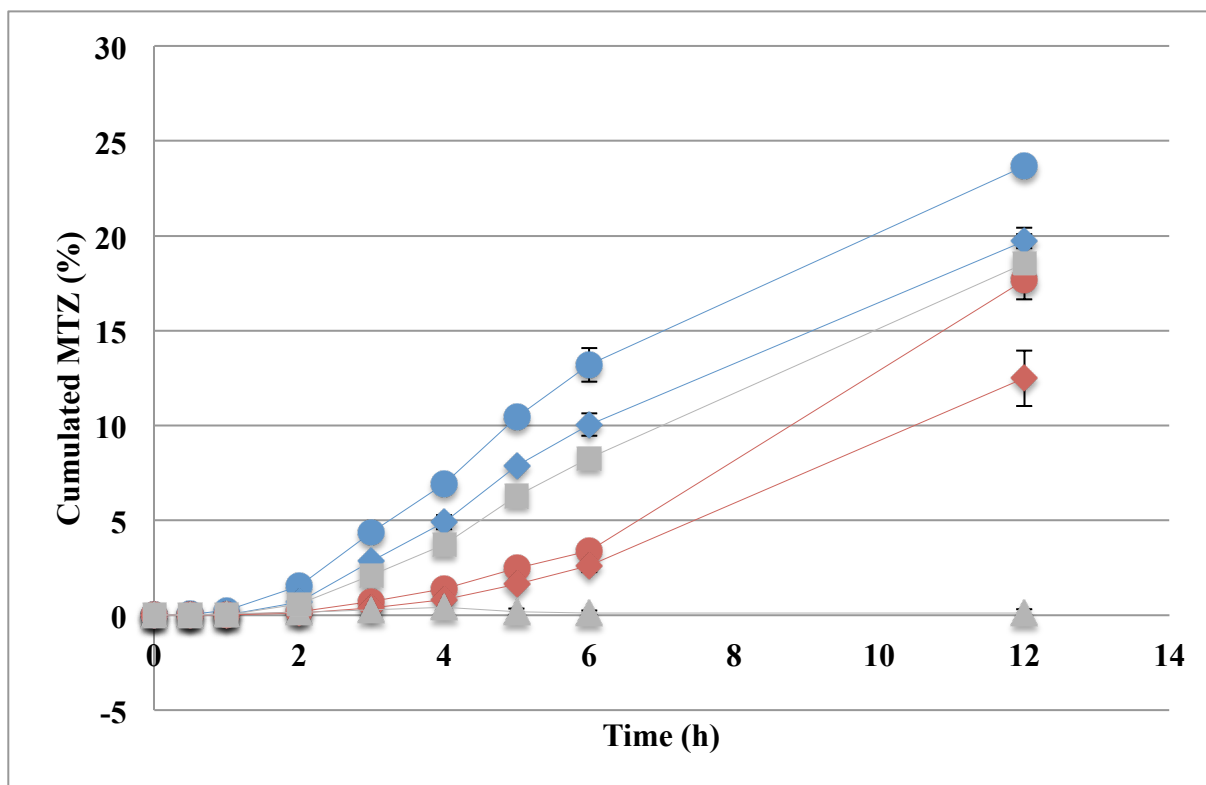


Figure 5. MTZ passage (in %) in function of time for: MTZ in solution in lactate buffer (■) chitosan-TBA+MTZ (●), chitosan+MTZ (◆), chitosan-TBA+MTZ jellified with F127 (20 wt%) (●) and chitosan+MTZ jellified with F127(20 wt%) (◆). SVF (▲) was used as negative control. MTZ was at a concentration of 7 mg/ml, chitosan was at 1% (w/w) and pluronic® F127 was at 20% (w/w). Data are the mean of three determinations ± sd. Low values of scale bars are included in the graphics can not be seen.

4.4 Evaluation of the trichomonacidal activity of the formulations

The trichomonacidal activity of the MTZ in solution and the hydrogel containing pluronic® F127 20wt%, chitosan 1 wt% with different concentrations of MTZ was studied after 24 h of direct contact with *T. vaginalis* (Fig. 6 and 7.). Control culture densities varied between 1 and 2.3 million.mL⁻¹ after 24 h and the results are expressed as % by average formula:

$$\frac{\text{number of } T.vaginalis \text{ by treated tube}}{\text{average number of control tubes}} \times 100.$$

After 24h of incubation, MTZ in solution in lactate buffer present a trichomonacidal activity. The IC₅₀ of MTZ was determined at a concentration of 0.75 µg/ml (Fig 6.).

After 24 h of incubation, even without MTZ, the hydrogel exhibited a trichomonacidal effect. The IC₂₀ was determined for a concentration of MTZ about 0.35 µg.mL⁻¹. From 0.75 to 5 µg.mL⁻¹ of MTZ a synergistic effect between the hydrogel and the MTZ was observed. In fact with lower concentration only the effect of the gel was observed with *T. vaginalis* viability between 20 and 26 % whereas from 0.75 µg.mL⁻¹ of MTZ *T. vaginalis* viability decrease about 7.3 % (Fig.7). Moreover this results are agree with the IC₅₀ of MTZ in solution determined precedently.

In order to complete the study, the trichomonacidal activity of the different components of the formulation was studied after 24 h of direct contact with *T. vaginalis* (Fig 8). Lactate buffer and chitosan did not exhibit any trichomonacidal activity. However pluronic F127 at 20 wt% had a significant activity against *T. vaginalis* with a viability of 51.6 % .

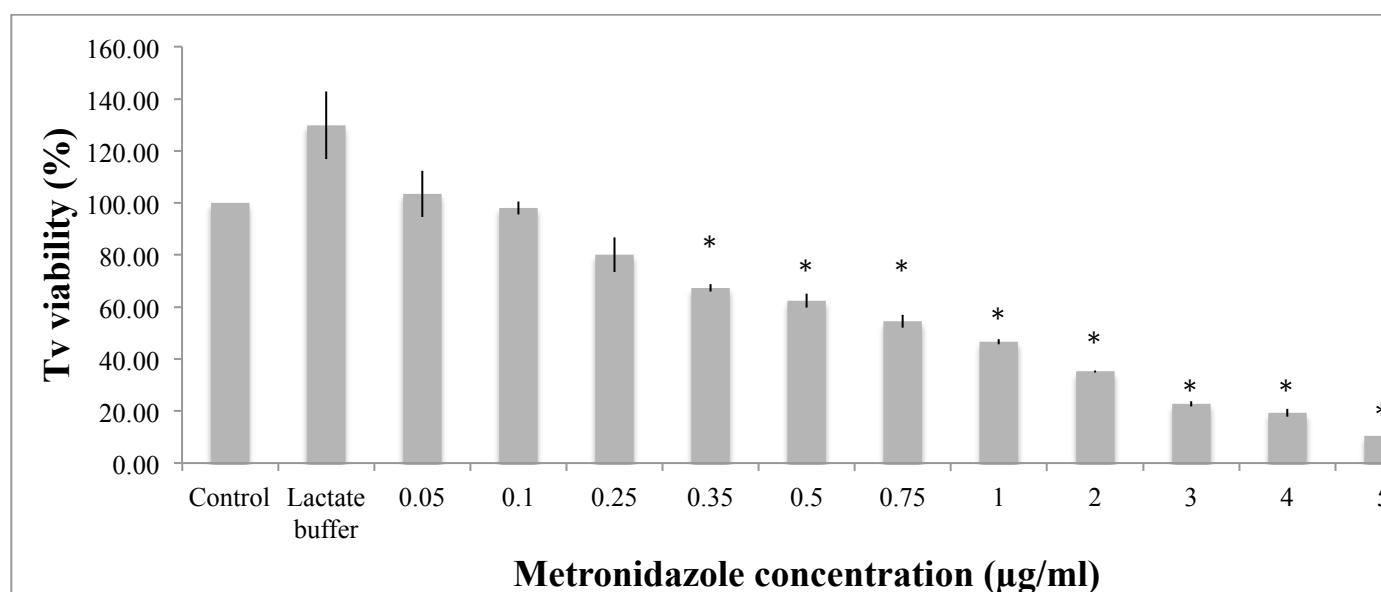


Figure 6. *T. vaginalis* viability percentage \pm sem in the presence of different concentrations of MTZ (from 5 to 0 µg/mL) in solution in lactate buffer pH 4.5. Presented data are the mean of three separate experiments. The viability percentage is determined relatively to controls. Culture medium (Control) was used as negative control. (* significant versus control $p < 0.05$).

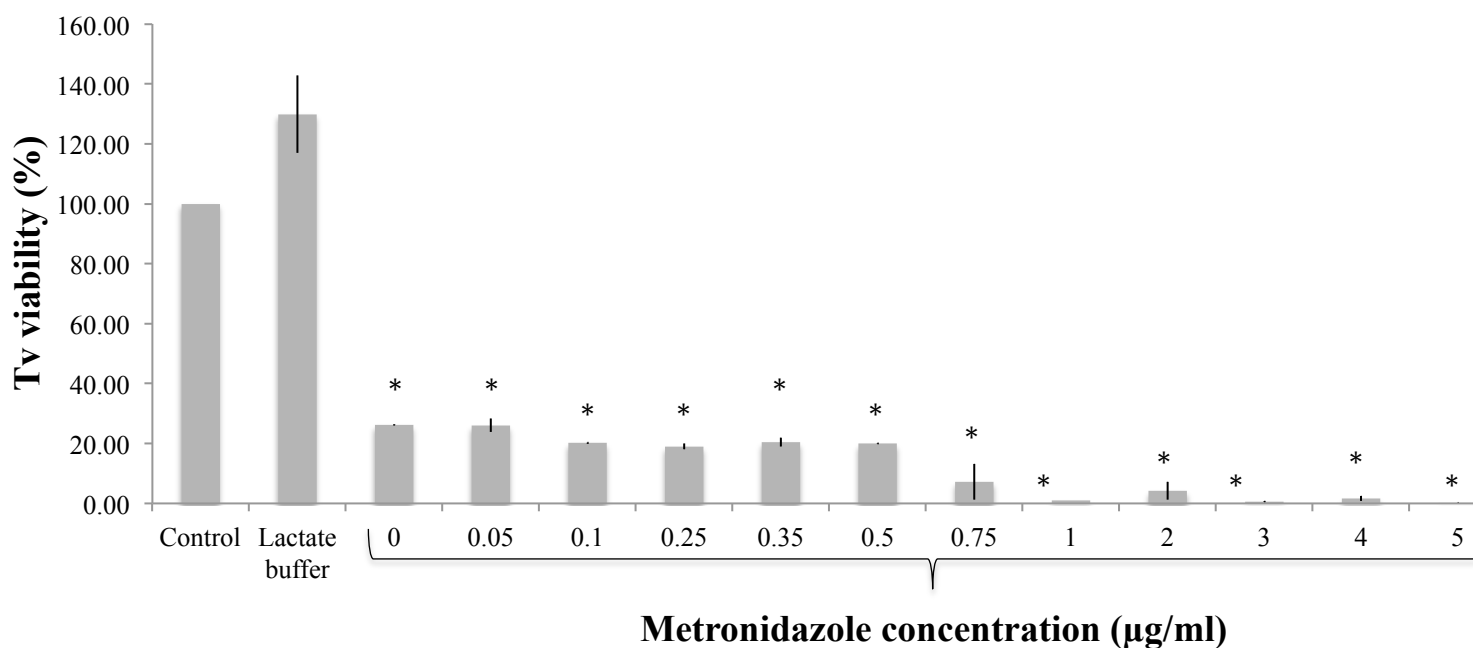


Figure 7. Optimization of MTZ concentration to get the best biological effect against *T. vaginalis*. *T. vaginalis* viability percentage \pm sem in the presence of final formulation containing chitosan (1 wt%), F127 (20 wt%) and different concentrations of MTZ (from 0 to 5 $\mu\text{g/mL}$). Presented data are the mean of three separate experiments. The viability percentage is determined relatively to controls. Culture medium (Control) was used as negative control. (* significant versus control $p < 0.05$).

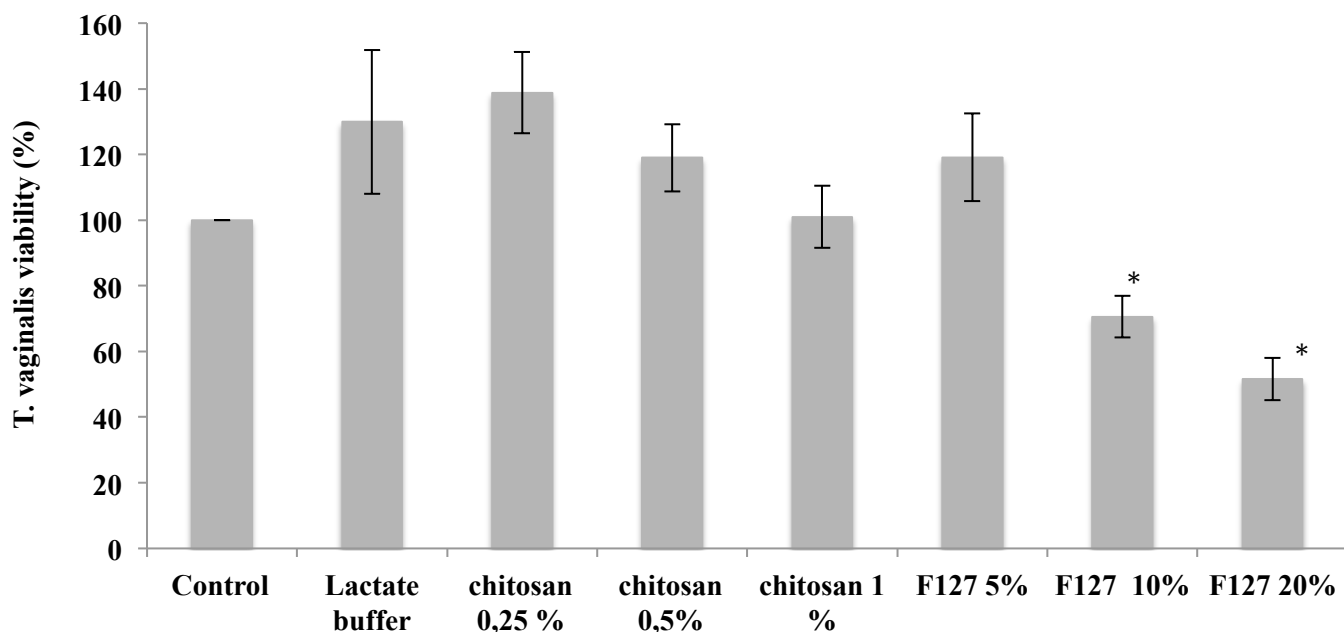


Figure 8. *T. vaginalis* viability percentage \pm sem in the presence of different concentrations of chitosan (from 0.25 to 1 wt%) and different concentrations of F127 (from 5 to 20 wt%). Presented data are the mean of three separate experiments. The viability percentage is determined relative to controls. Culture medium and lactate buffer were used as negatives controls. (* significant versus control $p < 0.05$).

4.5. Coculture HeLa/*T. vaginalis*

The trichomonacidal activity of the hydrogel containing pluronic[®] F127 20wt%, chitosan 1 wt% and different concentrations of MTZ was studied after 24 h of contact with *T. vaginalis* and HeLa cells (Fig. 9). After 24 h, no motile *T. vaginalis* was found with the formulation containing MTZ (0.7 mg.mL^{-1}). Moreover a significant trichomonacidal activity was observed with the chitosan and the pluronic[®] F127 combined. This activity was lower than with pluronic[®] F127 but can be explain by the positive charge of chitosan that may “coated” *T. vaginalis* that have a negative surface charge and protect them of F127. These results were in agreement with the previous ones and suggest a synergistic effect between the formulation and the MTZ. Checkerboard experiments according to the Odds method (2003) will be used to study the eventual sunergistic effect (Odds 2003).

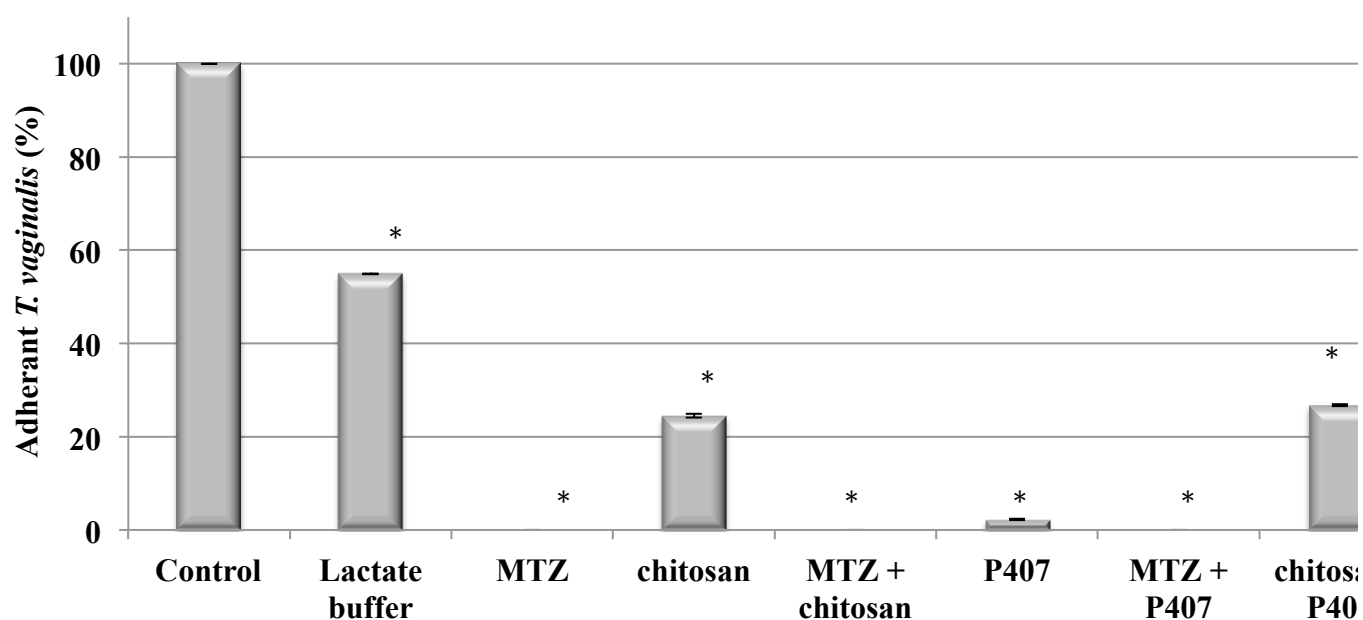


Figure 9. Effect of the formulation on the *Trichomonas vaginalis* adhesion on HeLa cells. MTZ was at a concentration of 7 mg/ml, chitosan was at 1% (w/w) and pluronic® F127 was at 20% (w/w). Culture medium and lactate buffer were used as negatives controls. Presented data are the mean of three separate experiments. (* significant versus control $p<0.05$) (* significant versus control $p<0.05$)

Conclusion

In conclusion, the CDs did not increase significantly the aqueous solubility of MTZ and they exhibit a toxicity against HeLa cells. Whereas the hydrogel containing 1 wt% of chitosan and 7 mg.mL⁻¹ of MTZ were not toxic against HeLa cells in absence of chitosan-TBA, increased the release time of MTZ and had an interesting *in vitro* trichomonacidal activity. Moreover the formulation without MTZ had shown a trichomonacidal effect and a synergic effect with the MTZ. This formulation is really interesting and will be a real alternative to oral therapy.

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Discussion générale

Discussion générale

1. Introduction

Le vagin de par sa physiologie et sa position anatomique est une bonne voie d'administration aussi bien pour l'application locale que systémique de médicaments. Cependant, la voie vaginale est très complexe, assez mal connue aujourd'hui et les formulations ne sont pas toujours adaptées aux besoins des femmes qui ressentent souvent un gêne majoritairement due aux écoulements de la forme médicamenteuse administrée. De plus, la voie vaginale est très intéressante pour la prévention et le traitement des IST et des MST.

De nombreuses IST comme les infections parasitaires ou fongiques sont traitées par voie systémique et peuvent provoquer des résistances aux principes actifs ainsi que des réactions allergiques importantes. C'est le cas notamment pour les infections parasitaires causées par *Trichomonas vaginalis* (*T. vaginalis*). C'est pour cette raison que nous avons décidé de mettre aux points des formulations pour une action locale sur ce parasite très répandu. L'idéal était que nos formulations répondent aux exigences de la voie vaginale notamment en termes de pH, de solubilité dans les fluides vaginaux et aux différences physiologiques dues à l'âge ou à la période dans le cycle menstruel. Nos formulations devraient également pouvoir être par la suite adaptées à différentes infections et pouvoir agir simultanément sur ces infections.

Pour optimiser l'efficacité des substances microbicides, il est toutefois nécessaire de disposer d'une formulation adaptée, capable de tapisser aisément la muqueuse vaginale tout en restant en place le plus longtemps possible. Pour atteindre ces objectifs, des formes mucoadhésives semblent être une bonne solution.

La bioadhésion peut être définie comme l'état dans lequel deux matériaux, dont l'un au moins est de nature biologique, sont maintenus ensemble par des forces interfaciales pendant un certain temps. Lorsque ce phénomène est dû au revêtement de mucus on parle de mucoadhésion. La cible des matériaux mucoadhésifs est le mucus qui est fortement visqueux et forme une couche protectrice sur la paroi externe des organes (das Neves et al. 2011). La mucoadhésion permet d'augmenter le temps de résidence de la formulation au niveau de la muqueuse et d'assurer ainsi une libération prolongée des principes actifs. Particulièrement, dans notre cas, la mucoadhésion permet d'assurer un rôle de barrière physique entre la muqueuse et les agents pathogènes de manière prolongée. Pour augmenter la mucoadhésion, il faut bien connaître la composition du mucus et pouvoir choisir des matériaux pouvant se lier à ce dernier par des liaisons plus ou moins fortes. C'est dans ce contexte que l'importance des gels qui semblent constituer la forme la mieux adaptée à un tel usage (das Neves, Amaral, and Bahia 2008).

Le but de notre étude a été de développer une formulation spécifiquement adaptée à une application locale au niveau vaginal.

Dans ce contexte, une formulation efficace doit respecter plusieurs exigences :

- Une fois administrée, elle doit tapisser aisément la muqueuse vaginale,
- Elle doit rester en place le plus longtemps possible par un phénomène de mucoadhésion, ce qui permettrait une libération prolongée dans le temps de l'agent anti-infectieux à une concentration efficace,
- Elle doit empêcher le parasite de diffuser jusqu'aux cellules épithéliales vaginales,
- Elle doit être peu toxique vis-à-vis du tissu vaginal,

- Lorsqu'une application locale est visée, il est préférable que le principe actif ne diffuse pas vers la circulation sanguine.

Pour atteindre ces objectifs nous avons utilisé trois principales stratégies :

- Nous avons souhaité augmenter la solubilité aqueuse apparente de différents principes actifs comme le métronidazole et l'albendazole qui sont des trichomonacides ainsi que le clotrimazole qui est un antifongiques particulièrement adaptés pour le traitement des candidoses. Pour cela nous avons utilisé différentes cyclodextrines.
- Nous avons formulé des systèmes nanoparticulaires pour l'administration de principes actifs au niveau de la muqueuse vaginale.
- Nous avons formulé des gels thermosensibles et mucoadhésifs capables de tapisser les muqueuses colonisées de façon à ce que la rémanence du principe actif à la surface des muqueuses soit la plus longue possible.

Compte tenu du fait qu'un bon développement préclinique contribue largement à éviter un échec clinique précoce, nous avons choisi d'effectuer une caractérisation approfondie de nos formulations avec ou sans principe actif. En effet, une telle approche est nécessaire afin d'amorcer le développement d'une forme microbicide topique efficace, non toxique et bien tolérée. Nous nous sommes donc fixés les objectifs suivants :

- 1/ Evaluer la solubilité aqueuse des principes actifs
- 2/ Formuler et caractériser d'un point de vue rhéologique les hydrogels thermosensibles et mucoadhésifs tout en tenant compte de l'influence de l'insertion de nanoparticules
- 3/ Etudier *in vitro* et *ex vivo* la toxicité des formulations
- 4/ Etudier la libération du métronidazole

5/ Vérifier *ex vivo* l'absence de passage du metronidazole à travers la muqueuse vaginale

6/ Etudier *in vitro* l'efficacité des formulations sur *Trichomonas vaginalis*.

2. Etudes préliminaires à la formulation

Pour répondre aux exigences de la voie vaginale, deux points nous ont paru essentiels au développement de notre formulation : l'étude de la toxicité et le contrôle du pH.

2.1 La toxicité

A chaque étape de la formulation, la toxicité de nos formulations a été étudiée ce qui nous a permis d'orienter l'étude. L'éthique et les dispositions réglementaires, se rapportant aux recherches touchant à l'humain, ont permis de mettre en place différents modèles pour étudier les effets d'agents thérapeutiques sur la muqueuse génitale. Ces modèles comportent bien entendu des modèles cellulaires, tissulaires, des systèmes de culture d'organes mais également des animaux expérimentaux. Pour étudier la muqueuse vaginale et plus particulièrement la mucoadhésion divers modèles animaux sont possibles. Par exemple, le lapin est le modèle le plus fréquemment utilisé pour l'étude des effets irritants sur la muqueuse vaginale (Zanelveld 1994). Cependant, parmi les plus grands animaux expérimentaux, le porc a l'avantage d'être remarquablement semblable à l'humain en terme d'anatomie, de physiologie, de métabolisme et de pathologie (Swindle and Smith 1998). Les similitudes du vagin porcin à celui de la femme en termes de pH, de sécrétions et de réponses inflammatoires ont conduit D'Cruz et coll. à proposer le porc comme un modèle pour les études d'irritation vaginale (D'Cruz, Erbeck, and Uckun 2005). Au niveau ultrastructurel,

l'épithélium du vagin de porcs ressemble étroitement à celui de la femme, y compris l'organisation des granules de recouvrement membranaire et les lamelles de lipides intercellulaires qui composent la barrière de perméabilité (Squier et al. 2008). De plus, le matériel porcin est aisément disponible dans les abattoirs locaux et peut être utilisé en moyenne dans les 3 heures suivant la mort de l'animal. Les restes tissulaires muqueux vitaux conservent durant ce laps de temps leur fonction de barrière car d'après plusieurs recherches ils seraient encore intacts 12 heures après l'abattage. Il a été également montré que la congélation n'affectait pas la couche de mucus ce qui permet de conserver la muqueuse avant utilisation (Squier et al. 2008).

Il existe différents modèles cellulaires utilisés pour étudier *in vitro* la muqueuse vaginale notamment les cellules d'épithélium vaginal et les cellules d'adénocarcinome du col de l'utérus HeLa. Cependant, les cellules d'épithélium vaginal sont des lignées primaires utilisables pour un nombre réduit d'essai. Les cellules HeLa quand à elles forment une lignée cellulaire immortelle facile à utiliser.

Pour ces raisons, nous avons décidé d'utiliser la muqueuse vaginale porcine comme modèle *ex vivo* et les cellules d'adénocarcinome du col de l'utérus HeLa comme modèle *in vitro* pour l'étude de nos formulations.

Pour l'étude de la toxicité des nanoparticules nous avons aussi utilisé des cellules d'adénocarcinomes de colon les Caco-2/TC7 et les HT-29/MTX. Les Caco-2/TC7 ont la particularité d'être différenciées et orientées contrairement aux HeLa, les HT-29/MTX sont elles aussi orientés et ont la particularité de produire du mucus. Ces deux modèles nous ont permis de comparer la toxicité des particules face à des cellules différentes et d'aller plus loin dans l'analyse tout en gardant à l'esprit que ce n'étaient pas des cellules vaginales.

2.2 Le contrôle du pH

Le pH est un élément très important dans l'équilibre de l'environnement vaginal. Cette valeur est maintenue par la flore résidente et principalement par les lactobacilles qui transforment le glycogène issu de l'épithélium exfolié en acide lactique. Les sécrétions menstruelles, le mucus cervical et le sperme peuvent augmenter le pH vaginal (Woolfson, Malcolm, and Gallagher 2000). De même, les infections peuvent modifier le pH du milieu vaginal, par exemple dans le cas des infections bactériennes, des trichomonoses ou des infections mixtes, le pH peut être fortement augmenté et peut atteindre la valeur de 7 (Anorlu et al. 2001; Sobel 2007). De plus, il a été montré que l'utilisation de formulation contenant un tampon acide pouvait traiter certaines vaginoses bactériennes (Andersch et al. 1986; Larsson and Forsum 2005).

Nous avons donc décidé de formuler nos hydrogels dans un tampon à pH 4.5. Dans un premier temps, nous avons utilisé un tampon acétate, couramment présent dans les formulations et qui est totalement compatible avec une application locale. De plus, l'acide acétique contenu dans ce tampon nous permettait de bien solubiliser le chitosane et notamment le chitosane thiolé qui n'est soluble qu'à un pH acide.

Puis nous avons décidé d'utiliser un tampon lactate plus approprié à la voie vaginale. En effet les gels à l'acide lactique sont utilisés en cas de sécheresse vaginale et ont montré une grande efficacité pour rétablir la flore vaginale en 10 à 15 jours après une infection bactérienne. L'acide lactique atténue les irritations des muqueuses de la vulve et du vagin et possède des propriétés régénérantes. De plus, l'utilisation d'un tampon lactate pourra permettre par la suite d'ajouter à notre formulation des probiotiques ce qui permettra de rééquilibrer la flore vaginale et d'augmenter l'efficacité du traitement. En effet, utilisés comme adjuvant au traitement standard avec des antibiotiques, des traitements alternatifs

comme des agents acidifiants ou des tampons et des probiotiques peuvent permettre de prévenir les réinfections à long terme (Verstraelen and Verhelst 2009). Par exemple, des probiotiques comme les lactobacilles utilisés en adjuvant produisent des substances bactéricides comme le peroxyde d'hydrogène et des tensioactifs qui jouent un rôle dans la prévention des infections (Boris and Barbés 2000).

3. Différentes stratégies pour l'amélioration de la biodisponibilité des principes actifs au niveau de la muqueuse vaginale

3.1. Augmentation de la solubilité aqueuse de différents principes actifs

Pour augmenter la solubilité aqueuse des principes actifs, différentes stratégies peuvent être envisagées, allant de l'utilisation des excipients pharmaceutiques à l'élaboration de nouvelles formulations (Luxenhofer et al. 2010; Mandal 2004) ou à la synthèse de pro-drogues (Golik, Matiskella, and Ueda 2001; Hernández-Luis et al. 2001; Mahfouz and Hassan 2001).

Une des stratégies très utilisées dans ce domaine s'avère être l'utilisation des cyclodextrines (CDs), ces dernières nous permettent d'éviter l'utilisation de co-solvants et de tensioactifs. En effet, une cyclodextrine est une molécule-cage d'origine naturelle qui permet d'encapsuler diverses molécules. En général, les CDs sont biocompatibles et non immunogéniques. Elles peuvent également protéger les molécules du milieu extérieur tout en

favorisant généralement leur biodisponibilité et leur libération prolongée au niveau du site de résorption (Del Valle 2004).

Les CDs (Fig. 1), découvertes par Villiers en 1891 (Villiers 1891) sont des oligosaccharides cycliques composés de sous-unités glucopyranose. Ces produits naturels sont obtenus par la dégradation enzymatique de l'amidon par la bactérie *Bacillus macerans*. On distingue trois familles de CDs naturelles appelées α -, β - et γ -CDs qui sont formées respectivement de 6, 7 et 8 unités de D-(+) glucopyranose (Fig. 1), mais il existe des familles de plusieurs dizaines de sous-unités qui ont été synthétisées. Le nombre d'unités de D-(+) glucopyranose détermine le diamètre de la cavité (4.7-5.3, 6.0-6.5 et 7.5-8.3 Å pour α -, β -, et γ -CD respectivement) (Fig. 2.b) et ainsi leur volume (174, 262 et 427 Å³ pour α -, β -, et γ -CD respectivement). Malheureusement, la β -CD qui est la plus utilisée pour l'incorporation de principes actifs est la moins soluble dans l'eau (18.5 g.L⁻¹ à 25 °C). De nombreux dérivés peuvent être obtenus à partir des cyclodextrines naturelles: les groupements hydroxyles des unités glucopyranose peuvent être aminés, estérifiés ou éthérifiés. L'alkylation des groupements hydroxyles de la β -CD a permis la synthèse de dérivés plus solubles dans l'eau tels que la méthyl- β -CD (Me- β -CD), l'hydroxypropyl- β -CD (HP- β -CD) et la sulfobutylether- β -CD (SBE- β -CD) (Brewster and Loftsson 2007).

Les unités de D-(+) glucopyranose sont liées entre elles par une liaison α -(1,4)-glucosidique, conférant aux CDs la forme d'un tronc cône creux, fortement hydrophile à l'extérieur et relativement hydrophobe à l'intérieur de la cavité. Cette structure singulière à double polarité confère aux CDs la capacité d'inclure dans leur cavité des molécules hydrophobes en formant un complexe d'inclusion (Fig. 2.c) permettant ainsi d'améliorer leur solubilité apparente en milieux aqueux (Loftsson and Duchene 2007).

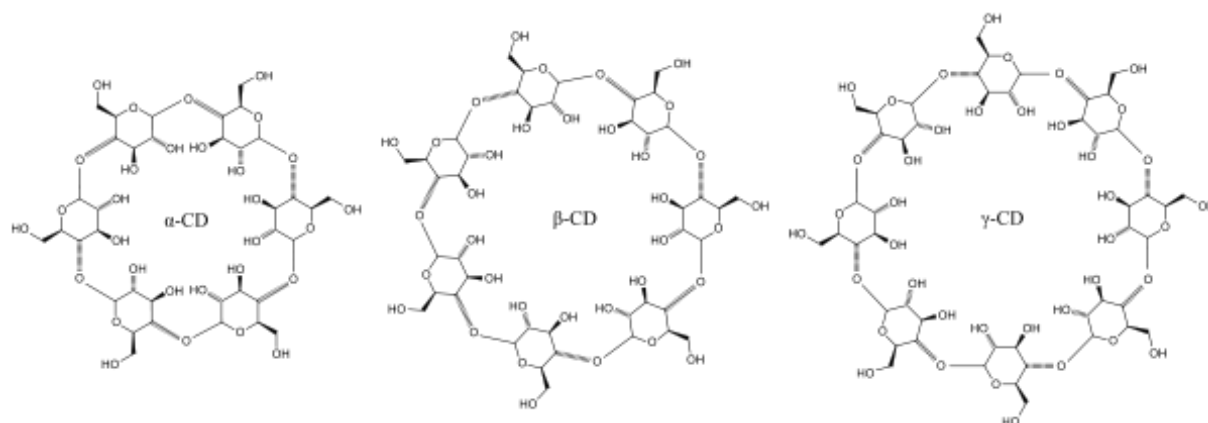


Figure 1: Structure de l' α -, β - et γ -cyclodextrines.

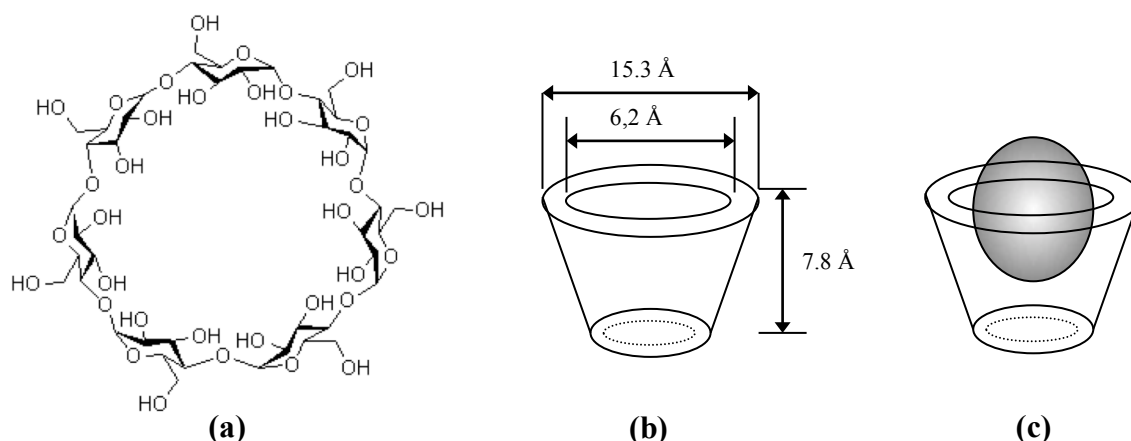


Figure 2: Formule chimique de la β -cyclodextrine (a) de taille et forme caractéristique (b) pouvant former un complexe d'inclusion avec un principe actif (c).

Bien que le travail de cette thèse soit orienté majoritairement sur l'étude de *T. vaginalis*, il nous a paru intéressant d'étudier en parallèle du métronidazole (MTZ) qui agit sur *T.vaginalis* d'autres principes actifs comme l'albendazole (ABZ) agissant lui aussi sur *T.vaginalis* et le clotrimazole (CTZ) qui est un antifongiques anti *Candida albicans*. En effet notre formulation ayant pour but de soigner un large panel de pathologies s'attaquant à la sphère vaginale, il

peut être intéressant de mixer différents principes actifs pour que notre formulation ait un large spectre d'activité.

Suivant la molécule les résultats, visant à l'augmentation de la solubilité, obtenus sont très différents. La concentration maximale de principe actif est obtenue pour les 3 molécules avec 40 wt% de méthyl- β -cyclodextrine (Me- β -CD). Cependant, avec cette même concentration en Me- β -CD, la solubilité aqueuse apparente du clotrimazole a été augmentée de près de 10 000 fois (de 0.00049 à 4.89 mg/ml), alors que celle de l'albendazole a été augmentée plus de 7 500 fois (de 0.0002 à 1.521 mg/ml) et celle du metronidazole seulement 10 fois (de 0,9 à 9 mg/ml).

Pour comprendre ces différences, l'analyse a été poussée plus en détail grâce à la RMN du proton en deux dimensions et à la modélisation moléculaire qui nous ont permis de mieux comprendre les mécanismes d'inclusion des principes actifs au niveau des cyclodextrines. La combinaison des études de solubilité, de la ^1H NMR-NOESY et des calculs de docking moléculaire ont permis d'identifier un mécanisme de liaison séquentielle bivalent entre le CTZ et la Me- β -CD. Une première molécule de Me- β -CD interagit avec les groupes phényle (en C14-C18 et C20-C24), puis, une seconde molécule de Me- β -CD interagit avec les deux chlorophényles (C9 et C10) et les groupes imidazole (C2, C4 et C5) du CTZ. Ces mêmes expériences ont permis de montrer que le bicycle central de l'ABZ était capable d'interagir avec la Me- β -CD en accord avec une stœchiométrie de type 1:1 (Tableau 1).

Cependant pour la RMN 2D, il s'est avéré que les déplacements du métronidazole étaient superposés à ceux de la cyclodextrine. Ceci a rendu impossible la détection des groupements du métronidazole qui auraient interagi avec la cyclodextrine.

Tableau 1 : Structures chimiques des différents principes actifs.

Molécules	Structures chimiques
Clotrimazole	
Albendazole	
Métronidazole	

Les études de toxicité ont montré que les formulations comportant 30 ou 40% de cyclodextrines étaient fortement toxiques envers les cellules HeLa notamment pour les complexes Me- β -CD/MTZ. Aux vu de ces résultats liés aux fait que la Me- β -CD n'augmentait pas spectaculairement la concentration aqueuse apparente du MTZ, il nous a semblé evident de ne plus utiliser de CDs pour la suite de notre étude.

3.2. Systèmes nanoparticulaires pour l'administration de PA au niveau de la muqueuse vaginale

La faible solubilité aqueuse de certaines molécules ne représente qu'une première problématique à résoudre pour améliorer la biodisponibilité des principes actifs une fois administrés. Il est aussi important d'augmenter le temps de résidence de ces molécules au niveau du site d'action et de leur assurer une libération prolongée.

Nous avons donc pensé qu'agir au niveau de la formulation pouvait s'avérer être une stratégie intéressante, en particulier avec la conception de formes nanoparticulaires mucoadhésives.

L'utilisation de nanoparticules pour encapsuler un principe actif présente différents avantages comme l'augmentation de la disponibilité et de la concentration des principes actifs ou encore l'augmentation du temps de contact au niveau de la zone d'action. Les nanoparticules de poly(cyanoacrylate d'isobutyl) (PIBCA) que nous avons utilisées sont composées d'un cœur hydrophobe de PIBCA et d'une couronne de chitosane. Nous nous sommes intéressés aux propriétés des systèmes constitués par des nanoparticules (NPs) cœur-couronne composées de PIBCA recouvertes de chitosane et de chitosane thiolé. Différentes études ont montré la capacité des NPs de poly(cyanoacrylate d'alkyle) (PACA) à encapsuler différentes molécules actives telles que des peptides (Delie and Blanco-Príeto 2005), comme l'insuline (Behan, O'Sullivan, and Birkinshaw 2002; Damge et al. 1997; Watnasirichaikul et al. 2000), des vaccins (Kreuter 1996), des agent anticancéreux (Mazzaferro, Bouchemal, Skanji, et al. 2012; Brigger, Dubernet, and Couvreur 2002; C. Vauthier et al. 2003), des agents anti-infectieux (Fawaz et al. 1997; Zimmer and Kreuter 1995; Xiong et al. 2014), des agents cytotoxiques ainsi que d'autres composés hydrophobes (Duan et al. 2010; Duan et al. 2012; Christine Vauthier et al. 2003).

Aujourd'hui, les NPs de PACA sont considérées comme des systèmes colloïdaux polymériques intéressants pour la délivrance de médicaments notamment pour les propriétés bioadhésives et biodégradables des PIBCA (I. Bravo-Osuna, Ponchel, and Vauthier 2007). Ainsi, la présence de chaînes de chitosan chargées positivement ainsi que la présence de groupements thiols sur la surface permettent aux NPs d'adhérer à la couche de mucus (Irene Bravo-Osuna et al. 2007; Bertholon, Ponchel, et al. 2006; Petit et al. 2012). De plus, les NPs de PACA semblent avoir un effet protecteur de la muqueuse intestinale vis-à-vis de la toxicité intestinale inhérente à un anticancéreux comme le docetaxel (Mazzaferro, Bouchemal, Maksimenko, et al. 2012).

Le chitosane est l'un des polysaccharides les plus utilisés dans le domaine biomédical et pharmaceutique grâce à ses propriétés intéressantes telles que sa biodégradabilité, sa biocompatibilité et sa faible toxicité (Ravi Kumar 2000). Le chitosane est un polysaccharide obtenu par déacétylation d'un polysaccharide naturel, la chitine qui est le deuxième polysaccharide le plus important et le plus abondant dans le monde après la cellulose. La chitine est la composante principale d'exosquelettes des arthropodes (crustacés) dont elle est principalement extraite. Mais ce polymère se retrouve également dans l'endosquelette des céphalopodes comme les calamars et les cuticules des insectes, la paroi de la plupart des champignons et dans certaines algues chlorophycées, levures et bactéries (Tolaimate et al. 2003). Le chitosane est caractérisé par sa masse molaire et par son degré de désacétylation (DDA) qui est en relation avec le rapport entre les groupements D-glucosamine et N-acétyl-glucosamine. Le chitosane a montré en tant qu'excipient, des caractéristiques intéressantes et une grande variété dans ces emplois. Il a la caractéristique d'être mucoadhésif en raison de l'interaction des charges positives portées par les amines primaires et des charges négatives portées par les mucines du mucus (Bonferoni et al. 2006; El-Kamel et al. 2002). Le chitosane est hydrophile, soluble dans les solutions acides (sa solubilité dépend de sa masse molaire et

de son DDA) et chargé positivement grâce à ses fonctions amines libres. Ces fonctions sont capables d'interagir notamment avec les cations divalents et ont permis la synthèse de plusieurs dérivés tels que les conjugués chitosane-EDTA, utiles pour surmonter la barrière enzymatique en vue de l'administration orale de peptides thérapeutiques (Andreas Bernkop-Schnürch and Krajicek 1998). Parmi les différents chitosanes modifiés, les chitosanes thiolés ou thiomers ont un intérêt particulier. En effet, la présence de groupements thiols, leur permet de former une liaison disulfure avec les sous-domaines des glycoprotéines du mucus riches en cystéine (Andreas Bernkop-Schnürch 2005; Irene Bravo-Osuna et al. 2006). Différents dérivés thiolés du chitosane ont été synthétisés : les conjugués avec l'acide thioglycolique, la cystéine, le 4-butyl-thioamidine (chitosane-TBA) (Andreas Bernkop-Schnürch, Guggi, and Pinter 2004; Andreas Bernkop-Schnürch, Hornof, and Guggi 2004; A Bernkop-Schnürch 2003), et les dérivés chitosane-thioethylamidine (Chito-TEA) (Kafedjiiski et al. 2006). Le chitosane thiolé que nous avons utilisé est le chitosane-TBA car il s'agit du chitosane thiolé le plus stable et qui présente les propriétés les plus intéressantes en termes de bioadhésion en comparaison avec les autres dérivés de chitosane thiolé (A Bernkop-Schnürch 2003).

Dans ce contexte, un de nos objectifs a été d'examiner la possibilité d'utiliser les NPs de PIBCA recouvertes de chitosane-TBA afin d'améliorer la biodisponibilité de principes actifs différents tout en respectant la muqueuse vaginale. Pour synthétiser les NPs, la technique choisie était la polymérisation anionique en émulsion. Cette technique est particulièrement intéressante car les NPs composées de copolymères amphiphiles sont obtenues en une seule étape. La polymérisation est rapidement et spontanément initiée par les fonctions nucléophiles du chitosane plutôt que par les ions hydroxydes de l'eau. La réaction est conduite à pH acide (pH ~ 1) (Bertholon et al. 2006) (Fig. 3).

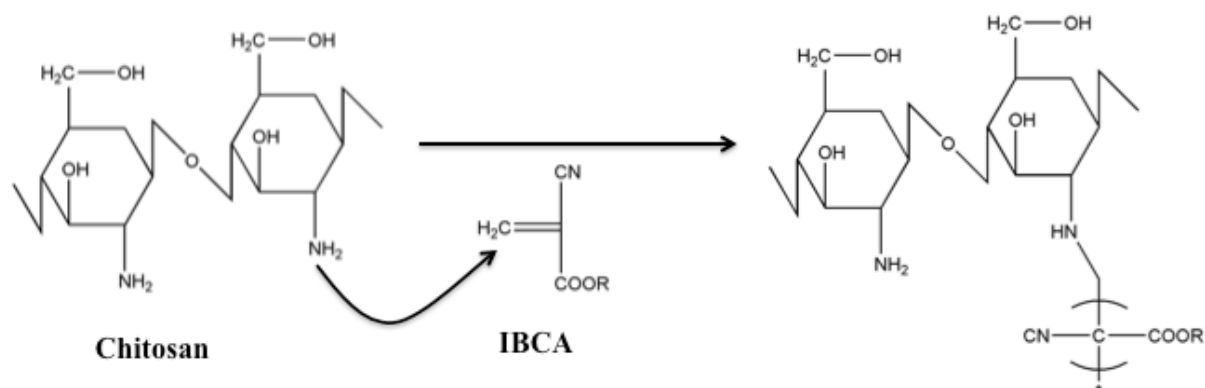


Figure 3 : Schéma de l'initiation de la polymérisation d'isocynoacrylate de butyl en présence de chitosan selon le mécanisme de polymérisation anionique en émulsions

Les études de toxicités ont montré que même à de très faible concentration les NPs présentaient une toxicité très élevée contre les cellules HeLa. Au contraire, ces mêmes concentrations n'induisent aucune toxicité apparente sur des modèles cellulaires intestinaux utilisés (Caco-2/TC7 et HT-29/MTX) et sur les coupes histologiques de vagin de porc. Ces résultats peuvent s'expliquer par le fait que les cellules HeLa ne sont pas polarisées contrairement aux deux autres modèles cellulaires. Par ailleurs, elles ne produisent pas de mucus qui pourraient les protéger contrairement aux cellules HT-29/MTX. Les cellules HeLa apparaissent donc beaucoup plus sensibles à la présence des nanoparticules de PIBCA que les souches de cellules différenciées testées et, par conséquent, moins aptes à résister à une quelconque toxicité causée par ces nanoparticules. La muqueuse vaginale porcine ne présente aucune lésion significative même en présence de fortes concentrations de NPs. Cette observation suggère que les NPs ne présentent pas de toxicité face à une muqueuse saine et en bonne santé recouverte d'une couche épaisse et régulière de mucus. En revanche, face à une muqueuse lésée et desquamée, les nanoparticules pourront induire une certaine toxicité puisque ces muqueuses comporteront plus de cellules indifférenciées et la couche de mucus pourra être moindre. Cette situation est notamment rencontrée dans le cas des infections où la

muqueuse présente quasiment toujours des lésions, desquame et sur laquelle la couche de mucus n'est plus homogène. Les résultats de notre étude de cytotoxicité des nanoparticules font apparaître qu'il est impensable d'utiliser les NPs de PIBCA pour soigner une muqueuse déjà lésée. Par ailleurs, elles ne peuvent pas non plus être proposées pour une utilisation dans un but préventif car, dans ce cas, aucune toxicité ne peut être tolérée.

La capacité de chargement des nanoparticules en MTZ s'est révélée être particulièrement faible. L'association aux nanoparticules ne permet pas d'atteindre les concentrations solubilisées dans l'eau. (tableau 2).

Tableau 2 : Concentration de MTZ encapsulée dans les NPs de PIBCA.

	Concentration du MTZ dans la solution totale (mg/ml)	Concentration du MTZ encapsulée dans les NPs (mg/ml)
NP vides	0.0 ± 0.0	0.0 ± 0.1
NP MTZ MebCD 30%	25.3 ± 0.5	0.1 ± 0.2
NP MTZ eau + HNO ₃ 0,2N	9.1 ± 0.1	1.8 ± 0.1

Les NPs de PIBCA vides ont présenté une activité trichomonacide importante. Ce résultat est intéressant et est en accord avec la toxicité obtenue sur les cellules HeLa qui sont également des cellules eucaryote. Ces résultats ont tendance à nous faire penser que l'activité trichomonacide n'est pas spécifique aux parasites. Cependant il a été montré que des NPs de PIBCA possédant une couronne de Dextran avaient une activité *in vitro* et *in vivo* anti *Trypanosoma brucei brucei* mais n'avaient aucune activité sur *T. vaginalis* et *Entamoeba histolytica* qui est un amibe pathogène du gros intestin (Lherm et al. 1987) Il a été montré que le chitosan composant la couronne ne possédait pas d'activité anti *T. vaginalis*. Il semblerait que cela soit l'association PIBCA/chitosan qui ait une activité contre *T. vaginalis* alors que le PIBCA serait cytotoxique pour les cellules HeLa. Pour vérifier cette hypothèse il faudrait

étudié ces phénomènes plus en détail notamment en étudiant les changements sur le chitosane lorsqu'il est sur la couronne des Nps ou encore en regardant l'effet du changement du polymère en couronne sur *T.vaginalis*.

Compte tenu de la toxicité potentielle mise en évidence des nanoparticules, de la très faible charge en principe actif qu'elles peuvent prendre ainsi que de la suspicion d'une activité trichomonacide non spécifique il apparaît évident que l'addition de nanoparticules de PIBCA chargées en MTZ n'apporteraient aucun bénéfice dans une formulation des gels microbicide destinés à la voie vaginale. La suite des travaux s'est donc orientée vers le développement et l'évaluation de formulations beaucoup plus simples constituées d'un gel thermosensible et mucoadhésif ne contenant pas de nanoparticules.

3.3. Conception et évaluation d'un hydrogel thermosensible et mucoadhésif

Pour répondre à la problématique de thermoreversibilité, les pluronics[®] semblent être de très bons candidats.

Les pluronics[®], également appelés poloxamères forment un groupe important de tensioactifs non ioniques de haute masse molaire et faiblement toxiques. Ils sont constitués d'une partie centrale hydrophobe de poly(oxyde de propylène) (POP) et de deux parties latérales hydrophiles de poly(oxyde d'éthylène) (POE communément appelés PEG) (Fig.4).

A une certaine concentration en poloxamère, les solutions aqueuses donnent des hydrogels thermoréversibles. Le phénomène de thermogélification est parfaitement réversible et est caractérisé par une température de transition sol-gel, également appelée température de gélification (Tgel). En-dessous de cette température, l'échantillon reste liquide et au-dessus, il devient semi-solide. La thermogélification résulte d'interactions entre des segments différents du

co-polymère (Dumortier et al. 2006; Pham Trong, Djabourov, and Ponton 2008). Avec l'augmentation de la température (T) et/ou de la concentration (C), les monomères constituant le poloxamère s'auto-associent en micelles grâce à la déshydratation des blocks de POP hydrophobes. Si on augmente encore la température et/ou la concentration, la micellisation est suivie de la gélification pour des échantillons assez concentrés. Diverses structures de gélifications ont été observées (Jeong, Kim, and Bae 2002; Zhang et al. 2013) (Fig. 5). Les hydrogels à base de poloxamère prolongent le temps de résidence au niveau du site d'application, ce qui améliore la biodisponibilité et l'efficacité du principe actif. En général, la toxicité des poloxamères est limitée (Johnston and Miller 1985).

Les poloxamères sous forme de monomère ou de micelles contiennent plusieurs fonctions hydroxyles (OH) libres. Ces fonctions permettent de créer des liaisons hydrogènes avec les composants du mucus, ce qui favorise la mucoadhésion.

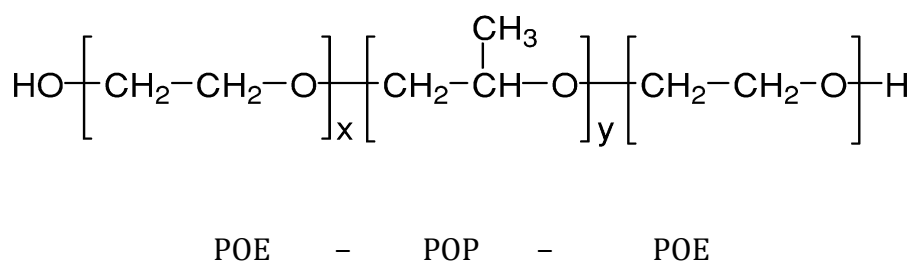


Figure 4 : Formule générale des pluronics®

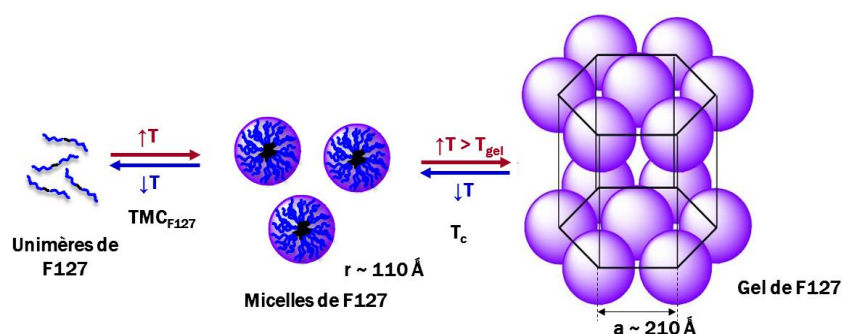


Figure 5 : Formation des micelles de pluronic® F127 puis des hydrogels à partir des unimères (Zhang et al. 2013).

En parallèle de l'étude de toxicité, nous avons mené une étude de caractérisation rhéologique et physico-chimique des hydrogels à base de pluronic[®] F127 ((EO)₉₇(PO)₆₉(EO)₉₇) avant et après inclusion de NPs de PIBCA vides. Il a été montré qu'après inclusion des NPs dans les hydrogels de pluronic[®] F127, les micelles étaient destabilisées et une partie de la réversibilité de la formation des micelles était perdue. Cependant, la température de gélification des hydrogels était maintenue en dessous de la température corporelle ce qui impliquait que le gel serait bien gélifié après application au niveau vaginal. En outre, les nanoparticules se sont montrées instables dans le gel et se dégradèrent en partie instantanément après ajout de pluronic[®]. Ces résultats ont conforté notre choix d'éliminer les nanoparticules de notre formulation. Il a donc été décidé de formuler des hydrogels beaucoup plus simples qui seraient totalement en accord avec une production industrielle à faible coût.

Il existe déjà sur le marché des gels vaginaux contenant du métronidazole à 0.75 % (MetroGel-Vaginal(R), 3M Pharmaceuticals, St. Paul, MN) (Wain 1998)

Cependant, ces gels ne sont pas thermoréversibles et n'ont pas besoin d'être conservés à 4°C, le métronidazole précipitant à cette concentration en présence de pluronic[®] F127 20%, il a donc été décidé de formuler des gels avec 0.70% de metronidazole car à cette concentration, il n'y a pas d'apparition de précipité ou de turbidité même à 4°C. Nous avons ensuite décidé d'ajouter du chitosane ou du chitosane-TBA à 1% à l'hydrogel pour favoriser la mucoadhésion.

L'ajout de ce chitosane n'a montré aucune modification rhéologique ni modifié les températures de gélification ou de micellisation des hydrogels contenant 20% de pluronic[®] F127.

Ces nouvelles formulations n'ont montré aucune toxicité significative envers les cellules HeLa excepté celles comportant du chitosane-TBA qui présentaient une toxicité limitée. De plus, les études de passage à travers la muqueuse vaginale porcine ont montré que les formulations gélifiées par le pluronic[®] limitaient le passage alors que les formulations contenant du chitosane-TBA le favorisait. Il a donc été décidé de n'utiliser que du chitosane non thiolé, qui lui, ne présentait aucune toxicité face aux cellules HeLa et favorisait ainsi une action locale du MTZ.

Il a également été montré que l'utilisation du gel de pluronic[®] favorisait la libération prolongée du MTZ. En effet, avec la formulation liquide, 100% du MTZ était immédiatement libéré. Seulement 68% du MTZ contenu dans l'hydrogel était libéré après 24h. Donc, la formulation hydrogel augmente de manière significative le temps de libération de MTZ.

Le plus intéressant est que ces hydrogels dans lesquels le MTZ est dissous ont montré une surprenante activité *in vitro* sur les *T. vaginalis* tout en montrant un effet protecteur sur les cellules HeLa dans le cas de co-cultures de *T. vaginalis* et des cellules HeLa. En effet, en absence de traitement et en présence de *T. vaginalis*, les cellules HeLa étaient totalement décollées alors qu'en présence de traitement après 24h le tapis cellulaire était intact. De plus, en présence d'hydrogel ne contenant pas de MTZ, le tapis cellulaire des cellules HeLa est moins abimé et ne se décolle pas totalement ce qui prouve un effet protecteur de l'hydrogel seul et confirme l'effet barrière attendu.

4. Conclusion

La formulation de départ qui consistait à solubiliser des principes actifs à l'aide de CDs puis inclure ces complexes CDs/Principe actifs dans un système nanoparticulaire qui serait lui

même inclu dans un hydrogel thermosensible était totalement compatible avec une production pharmaceutique et prometteur. Cependant, les études de toxicité ont montré dans un premier temps que les CDs à des concentrations élevées étaient très toxiques envers les cellules HeLa et il en était de même pour les NPs de PIBCA. Bien que les NPs ne présentent pas de toxicité significative ni sur des modèles cellulaires intestinaux (Caco-2/TC7 et HT-29/MTX) ni sur les coupes histologiques de vagin de porc, il nous a semblé évident d'abandonner ces pistes de recherche. En effet, nous avons pour but de trouver une formulation modèle pour la voie vaginale qui ne présente aucune toxicité. Nous avons donc orienté le travail sur un hydrogel beaucoup plus simple contenant directement du MTZ. Ce gel s'est montré très performant envers *T. vaginalis*, a permis d'augmenter le temps de libération du MTZ tout en ne montrant aucune toxicité envers les cellules HeLa et même en ayant un effet protecteur sur ces dernières en les protégeant des *T. vaginalis*. Notre démarche nous a donc permis de développer un gel très prometteur. Des études complémentaires sont nécessaires pour qu'il soit totalement opérationnel et réponde à toutes les exigences de la voie vaginale mais les résultats précédemment obtenus sont très encourageant.

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Conclusions et perspectives

Conclusions et perspectives

Face aux nombreux défis que présentent les infections vaginales dans le monde, il est urgent de mettre au point des stratégies efficaces notamment en matière de prévention et de traitement. Protéger les femmes contre l'infection revient à protéger plus de la moitié de la population mondiale. De plus, pour un ensemble de raisons sociales et psychologiques, un moyen de prévention uniquement contrôlé par celles-ci, sans le besoin d'y associer leur partenaire semble être une alternative à privilégier. En outre, une formulation efficace qui permettrait une libération prolongée des principes actifs et permettrait de réduire la fréquence d'application voir de traiter en une seule application et augmenterait ainsi l'observance des patients et limiterait ainsi l'apparition des résistances thérapeutiques due dans la majorité des cas à un mauvais usage des traitements.

Au cours de notre étude nous avons montré qu'une formulation simple et totalement en accord avec une production industrielle peu coûteuse pouvaient être efficace contre *T. vaginalis*. Ces formulations ont montré son efficacité à (i) libérer progressivement le métronidazole et assurer ainsi une libération prolongée de la molécule ; (ii) tuer les *T. vaginalis* in vitro en milieu liquide et en co-culture avec des cellules HeLa et (iii) protéger les cellules HeLa de l'activité des *T. vaginalis*.

Ces formulations efficaces contre *T. vaginalis* forment une barrière physique et chimique contre les parasites sur un plan curatif. De plus elles sont très bien tolérées par les cellules HeLa qui sont un modèle fragile qui mime parfaitement les cellules desquamées après une infection. Dans le futur, pour évaluer l'action préventive que pourrait avoir cet hydrogel, il faudrait faire des co-cultures HeLa/*T. vaginalis* en plaçant le gel en premier sur le tapis de cellules HeLa puis y ajouter les *T. vaginalis* et étudier simultanément la viabilité des cellules

et des parasites. Pour être au plus proche de la réalité un modèle biomimétique d'écosystème sain et infecté pourrai être imaginé avec de la muqueuse vaginale porcine.

La formulation de l'hydrogel thermosensible développée au cours de ce travail ayant montré une bonne efficacité contre *T. vaginalis*, nous avons poursuivi nos travaux dans le but de développer cet hydrogel comme une plateforme destinée à délivrance d'un traitement local d'anti-infectieux au niveau de la voie vaginale et diriger contre d'autres agents infectieux : le clotrimazole et l'albendazole. Les travaux menés dans cet objectif ont mis en évidence une difficulté liée aux propriétés de solubilité des principes actifs. Le metronidazole utilisé contre *T. vaginalis* présente une assez bonne solubilité aqueuse ce qui permet de l'incorporer directement dans l'hydrogel de Pluronic® F127 à une concentration suffisante pour produire une bonne activité anti-parasitaire. Pour les deux autres molécules considérées, l'albendazole, activité anti *T. vaginalis*, et le clotrimazole, activité anti *C.albicans*, leur solubilité aqueuse quasi nulle ne permet pas d'incorporer dans la formulation des concentrations suffisantes pour avoir une activité anti-infectieuse. L'utilisation de cyclodextrine et de nanoparticules de poly(cyanoacrylate d'isobutyl) permet de charger l'hydrogel avec des concentrations en principe actif intéressantes sans modifier de manière notable les propriétés rhéologiques et de gélification de l'hydrogel. Cependant, cette augmentation de charge en principe actif de l'hydrogel se fait au détriment de la tolérance de la formulation par les cellules et les tissus ce qui n'est évidemment pas acceptable. D'autres vecteurs destinés à augmenter la solubilité de ces molécules devront être envisagés. Leur choix pourrait être porté vers d'autres formes nanoparticulaires constitués par de polymères mieux tolérés que les poly(cyanoacrylate d'alkyl). Certaines nanoparticules notamment comme les solide-lipid-nanoparticule (SLN) ont

montrés leur efficacité contre *Candida albicans* tout en ne présentant aucune toxicité envers les cellules HeLa.

En parallèle à ces études sur l'incorporation d'autres principes actifs anti-infectieux dans l'hydrogel, il sera utile d'étudier de manière précise le pouvoir mucoadhésif de l'hydrogel, l'évolution de son pH au cours de temps dans des conditions reflétant l'utilisation et enfin, l'effet sur la viabilité des *T. vaginalis* devra être précisé sur le plan de la durée de d'action de l'activité antiparasitaire de la formulation.

RESUME

Selon les dernières estimations de l'OMS, on enregistre chaque année dans le monde 498,9 millions de nouveaux cas d'infections sexuellement transmissibles (IST) dont 276,4 millions sont dus au parasite *Trichomonas vaginalis* (*T. vaginalis*). Au niveau du tractus génital, la colonisation et l'irritation de la muqueuse vaginale par *T. vaginalis* favorisent la survenue de complications infectieuses. Ces infections associées peuvent conduire à des infections chroniques et avoir à terme des conséquences graves (stérilité, rupture prématuré du placenta, mort prématurée du nourrisson). De plus, ces infections vaginales représentent des facteurs qui favorisent les infections par le Virus d'Immunodéficience Humaine (VIH-1).

A l'heure actuelle, la lutte contre ce type d'infections consiste à agir tant au niveau curatif, que préventif. Ainsi, l'objectif de ce projet est de développer de nouvelles formulations pour la prévention et le traitement des pathogènes qui colonisent les muqueuses vaginales. Dans ce contexte, la formulation que nous proposons est composée de metronidazole inclut dans un hydrogel thermogélifiant à base de pluronic® F127 et de chitosane.

Il a été montré que cet hydrogel conserve ces propriétés physiques à une température physiologique même après dilution dans les fluides vaginaux. Ces hydrogels sont stables et permettent une libération prolongée du metronidazole. La formulation n'a montré aucune toxicité envers les cellules HeLa ni envers la muqueuse vaginale porcine. L'efficacité de cette formulation a été prouvée envers *T. vaginalis* et présente un effet protecteur envers les cellules HeLa en présence de *T. vaginalis*. L'ensemble des résultats suggère donc la capacité de cette formulation à constituer une double barrière, physique et pharmacologique, protectrice de la muqueuse vaginale vis-à-vis de *T. vaginalis*.

MOTS CLES : Systèmes thermogélifiants, hydrogel, pluronic® F127, métronidazole, albendazole, clotrimazole, *Trichomonas vaginalis*, *Candida albicans*, cyclodextrines, nanoparticules polymériques, profils de libération, mucoadhésion, voie vaginale.

LABORATOIRE DE RATTACHEMENT :

UMR CNRS 8076 – Laboratoire de Chimiothérapie antiparasitaire.

5, Rue Jean Baptiste Clément

92296 Châtenay-Malabry, France

POLE : MICROBIOLOGIE ET THERAPEUTIQUE ANTI-INFECTIEUSE

UNIVERSITE PARIS-SUD 11

UFR « FACULTE DE PHARMACIE DE CHATENAY-MALABRY »

5, Rue Jean Baptiste Clément

92296 Châtenay-Malabry, France